The E3 Ubiquitin Ligase Atrophin Interacting Protein 4 Binds Directly To The Chemokine Receptor CXCR4 Via a Novel WW Domain-mediated Interaction

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The E3 ubiquitin ligase atrophin interacting protein 4 (AIP4) mediates ubiquitination and down-regulation of the chemokine receptor CXCR4. AIP4 belongs to the Nedd4-like homologous to E6-AP carboxy terminus domain family of E3 ubiquitin ligases, which typically bind proline-rich motifs within target proteins via the WW domains. The intracellular domains of CXCR4 lack canonical WW domain binding motifs; thus, whether AIP4 is targeted to CXCR4 directly or indirectly via an adaptor protein remains unknown. Here, we show that AIP4 can interact directly with CXCR4 via a novel noncanonical WW domain-mediated interaction involving serine residues 324 and 325 within the carboxy-terminal tail of CXCR4. These serine residues are critical for mediating agonist-promoted binding of AIP4 and subsequent ubiquitination and degradation of CXCR4. These residues are phosphorylated upon agonist activation and phosphomimetic mutants show enhanced binding to AIP4, suggesting a mechanism whereby phosphorylation mediates the interaction between CXCR4 and AIP4. Our data reveal a novel noncanonical WW domain-mediated interaction involving phosphorylated serine residues in the absence of any proline residues and suggest a novel mechanism whereby an E3 ubiquitin ligase is targeted directly to an activated G protein-coupled receptor.

INTRODUCTION

The chemokine receptor CXCR4 and its cognate ligand stromal cell-derived factor (SDF)-1 α (Bleul *et al.*, 1996; Oberlin *et al.*, 1996), also termed CXCL12 (Murphy, 2002), have critical functions in cardiogenesis, vasculogenesis, neural development, stem cell homing, and leukocyte chemotaxis among others (Nagasawa *et al.*, 1996; Tachibana *et al.*, 1998; Zou *et al.*, 1998; Lu *et al.*, 2002). Dysregulation of CXCR4 signaling, expression, or both is associated with several pathological conditions, including cardiovascular disease, cancer, and warts, hypogammaglobulinemia, recurrent infection, and myelokathexis (Damas *et al.*, 2000; Muller *et al.*, 2001; Balkwill, 2004; Diaz and Gulino, 2005; Walter *et al.*, 2005). Thus, elucidating the mechanisms that regulate CXCR4 is critical for understanding its contribution to these pathologies and for developing new strategies to manipulate receptor signaling to treat diseases associated with CXCR4 dysregulation.

CXCR4 is a G protein-coupled receptor (GPCR) that couples to G α_i to elicit a variety of cellular signaling responses (Busillo and Benovic, 2007). Similar to other GPCRs, signaling by CXCR4 is rapidly desensitized at the cell surface,

followed by internalization into endosomes in which it is sorted to lysosomes for degradation. The posttranslational modification of CXCR4 with ubiquitin, a 76-amino acid polypeptide, is critical for regulation of CXCR4 endocytic trafficking (Marchese and Benovic, 2001). We have shown previously that CXCR4 ubiquitination on carboxy-terminal tail (C-tail) lysine residues targets the receptor for lysosomal degradation (Marchese and Benovic, 2001; Marchese *et al.*, 2003). Ubiquitination of activated CXCR4 is mediated by the E3 ubiquitin ligase atrophin interacting protein 4 (AIP4) at the plasma membrane (Marchese and Benovic, 2001; Marchese *et al.*, 2003). However, the mechanism by which AIP4 is targeted to activated CXCR4 remains unknown.

AIP4 is a member of the Nedd4-like family of E3 ubiquitin ligases and ubiquitinates a diverse set of proteins involved in a variety of cellular processes (Ingham *et al.*, 2004; Shearwin-Whyatt *et al.*, 2006). AIP4 is composed of an amino-terminal C2 domain, a proline-rich region (PRR), four tandemly linked WW domains, and a carboxy-terminal catalytic homologous to E6-AP carboxy terminus (HECT) domain. C2 domains are phospholipid binding domains that may also mediate protein-protein interactions (Cho and Stahelin, 2006). The PRR has been shown to bind to Src homology 3 domains (Angers *et al.*, 2004; Janz *et al.*, 2007). WW domains, named after two conserved tryptophan residues, are ~35–45 amino acid residues in length and are structurally similar; they can be divided into four distinct groups based on their ability to interact with proline-rich sequences in various contexts (Macias *et al.*, 2002; Hu *et al.*, 2004; Ingham *et al.*, 2005a). Typically, the WW domains mediate binding of the Nedd4-like family members with their targets either directly or indirectly through a protein intermediate (Ingham *et al.*, 2004; Shearwin-Whyatt *et al.*, 2006). All four WW domains of AIP4 have been shown to bind to PY motifs

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Abbreviations used: AIP4, atrophin-interacting protein 4; FRET, fluorescence resonance energy transfer; GPCR, G protein-coupled receptor; GST, glutathione transferase; HECT, homologous to E6-AP carboxy terminus; TIRF, total internal reflection fluorescence.

(e.g., PPxY, PPPY) within their target proteins and/or peptides containing PY motifs (Winberg *et al.*, 2000; Ingham *et al.*, 2005b). The HECT domain is a conserved ~350-amino acid catalytic domain that contains an active site cysteine residue that forms a direct thiolester bond with ubiquitin before its transfer to target proteins (Huibregtse *et al.*, 1995; Pickart and Eddins, 2004).

The molecular determinants responsible for targeting the ubiquitination machinery to GPCRs remain poorly understood. CXCR4 was the first mammalian GPCR shown to be ubiquitinated by a Nedd4-like E3 family member (Marchese *et al.*, 2003). In yeast cells, the α -mating factor receptor (Ste2), a GPCR, is ubiquitinated by Rsp5, the yeast ortholog of Nedd4-like E3s (Dunn and Hicke, 2001). Genetic and biochemical studies have revealed that the Rsp5 WW domains are required for ubiquitination of the receptor, suggesting that the WW domains are involved in receptor recognition. However, the C-tail of Ste2 lacks any known WW recognition motifs, suggesting that Rsp5 is recruited to the receptor via an indirect mechanism, possibly via an intermediate protein that is yet to be identified (Dunn and Hicke, 2001). As with Ste2, the intracellular domains of CXCR4 do not contain obvious WW domain recognition sequences, but whether an adaptor protein is required for AIP4 recruitment to the receptor remains to be determined. Interestingly, studies of the mammalian β_2 -adrenergic receptor (β_2 AR), a prototypic GPCR used widely for studying GPCR signaling and trafficking (Pierce *et al.*, 2002), implicate the endocytic adaptor protein arrestin-3 in the recruitment of the E3 ubiquitin ligase Nedd4-1 to the receptor (Shenoy *et al.*, 2001, 2008). Arrestins have been implicated in the regulation of CXCR4 signaling and trafficking (Orsini *et al.*, 1999; Cheng *et al.*, 2000); however, recently, we have shown that arrestins are not involved in CXCR4 ubiquitination (Bhandari *et al.*, 2007). Thus, whether AIP4 targeting to CXCR4 involves the WW domains either through a direct or indirect interaction and/or possibly other domains remains to be determined. Here, we report that AIP4 can bind directly to CXCR4 via an interaction between CXCR4 C-tail serine residues and the WW domains of AIP4 revealing, a novel mechanism whereby an E3 ligase is directly targeted to a GPCR.

MATERIALS AND METHODS

Cell Lines, Reagents, and Antibodies

Human embryonic kidney (HEK) 293 cells (Microbix, Toronto, ON, Canada) were maintained in DMEM (Mediatech, Herndon, VA) supplemented with fetal bovine serum (FBS; HyClone Laboratories, Logan, UT). The anti-hemagglutinin (HA) monoclonal antibodies (mAbs) and polyclonal antibodies were purchased from Covance (Richmond, CA). The anti-FLAG M2-horse-radish peroxidase (HRP) mAb and λ protein phosphatase were from Sigma-Aldrich (St. Louis, MO). The anti-HIS antibody was from QIAGEN (Valencia, CA). The anti-AIP4 polyclonal (D-20) and monoclonal (G11) antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA), whereas the anti-glutathione transferase (GST) antibody and glutathione-Sepharose 4B resin were from GE Healthcare (Little Chalfont, Buckinghamshire, United Kingdom). The anti- β -tubulin mAb was from Accurate Chemical & Scientific (Westbury, NY), and the anti-actin antibody was from MP Biomedicals (Solon, OH). Horse anti-mouse and anti-goat antibody horseradish conjugates and VECTASHIELD mounting medium for immunofluorescence were from Vector Laboratories (Burlingame, CA). The Alexa-Fluor 594 goat anti-mouse conjugate was from Invitrogen (Carlsbad, CA). Stromal cell-derived factor (SDF)-1 α (a.k.a. CXCL12) was from PeptoTech (Rocky Hill, NJ). Calf intestinal alkaline phosphatase (CIP) was from New England Biolabs (Ipswich, MA).

DNA Constructs

HA-tagged CXCR4, FLAG-AIP4, and FLAG-ubiquitin were as described previously (Marchese *et al.*, 2003). GST-fusion constructs of the four individual AIP4 WW domains were as described previously (Marchese and Benovic, 2004) and kindly provided by Dr. Anthony Pawson (Samuel Lunenfeld Research Institute, Toronto, ON, Canada). The HA-tagged serine mutants of

CXCR4 in pcDNA3 were generated by the polymerase chain reaction (PCR) by using HA-CXCR4 WT as the template. For GST-fusion constructs of the C-tail, wild-type, S324/5A, and S324/5D CXCR4 were used as templates to amplify amino acid residues 308–352 by PCR and cloned in-frame to GST by using the pGEX-4T2 bacterial expression vector. For GST-fusion constructs of AIP4, wild-type AIP4 (amino acids 2–862), and WW domains I–IV (wild-type and mutant constructs; amino acids 260–486) were amplified by PCR and cloned into the BamHI and XhoI sites of pGEX-4T2 to generate full-length GST-AIP4 and GST-WW-I–IV. Individual point mutants of AIP4 were made in the context of GST-WW-I–IV to include Q297A, N329A, Q297A/N329A, W313A, W345A, W313A/W345A, and Q297A/N329A/W313A/W345A (4A). To create amino-terminally tagged His-AIP4 and His-WW-I–IV (wild-type and point mutants), full-length AIP4 (amino acids 2–862) and WW domains I–IV (amino acids 260–486) were amplified by PCR and subcloned into the BamHI-PvuII and BamHI-XhoI sites of pRSET-A (Invitrogen), respectively. The HA-CXCR4-YFP construct (described in Bhandari *et al.*, 2007) was used as the template to clone the HA-S324/325A-yellow fluorescent protein (YFP) mutant. Full-length AIP4 was amplified and subcloned into the KpnI-BamHI sites of pECFP-N1 (Clontech, Mountain View, CA) plasmid to generate AIP4-cyan fluorescent protein (CFP). The integrity of all the constructs was verified by sequencing.

GST-Fusion Protein Expression and Binding Assay

Escherichia coli BL21 cells transformed with GST-fusion constructs were grown overnight at 37°C. The next day, cultures were diluted 1:50 and grown to an OD₆₀₀ ~ 0.4 at 37°C and then induced with 0.1 or 0.5 mM isopropyl β -D-thiogalactoside (IPTG) for 1–3 h at 18°C. Cells were pelleted and resuspended in binding buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% Triton X-100, 1 mM dithiothreitol, 10 μ g/ml leupeptin, 10 μ g/ml pepstatin A, and 10 μ g/ml aprotinin), and sonicated on ice. Lysates were clarified by centrifugation at 21,000 $\times$ g for 20 min at 4°C, followed by incubation with glutathione-Sepharose 4B resin for 1 h at 4°C while rocking, and then they were washed and resuspended in binding buffer. For binding experiments, GST-fusion proteins (~0.3–1 μ g) were incubated with cell lysates prepared from HEK293 cells expressing the protein of interest or purified His-tagged proteins for 4–16 h. Samples were washed in binding buffer, eluted in 2 $\times$ sample buffer for 30 min at room temperature and analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) followed by Western blotting.

Expression and Purification of 6 $\times$ His-AIP4

E. coli BL21-DE3 cells, transformed with 6 $\times$ His-AIP4- and 6 $\times$ His-WW-I–IV-pRSET-A were grown to an OD₆₀₀ 0.3–0.5 and induced with 1.0 mM IPTG for 1–4 h at 18°C. Cells were pelleted and resuspended in lysis buffer (for 6 $\times$ His-AIP4, the buffer used was 50 mM phosphate buffer, pH 7.4, 300 mM NaCl, and 10 mM imidazole; for 6 $\times$ His-WW-I–IV the buffer used was 50 mM phosphate buffer, pH 8.0, 500 mM NaCl, 10 mM imidazole, and 0.1% Triton X-100) containing protease inhibitors (aprotinin, pepstatin A, and leupeptin, each at 10 μ g/ml), sonicated on ice, and clarified by centrifugation. Clarified lysates containing 6 $\times$ His-AIP4 were applied to pre-equilibrated nickel-nitrilotriacetic acid mini columns, washed (wash buffer: 50 mM phosphate buffer, pH 7.4, 300 mM NaCl, and 30 mM imidazole), and eluted (elution buffer: 50 mM phosphate buffer, pH 7.4, 300 mM NaCl, and 300 mM imidazole), according to the manufacturer's instructions (ProPur IMAC Mini spin column kit; Nalge Nunc International, Rochester, NY). Clarified lysates containing 6 $\times$ His-WW-I–IV (WT and point mutants) were incubated with equilibrated His-Select Ni-affinity beads (Sigma-Aldrich) for 1 h at 4°C, washed, and eluted in the same buffer as the lysis buffer containing 100–500 mM imidazole. The binding assay between 6 $\times$ His-AIP4 (500 ng) or various amounts of 6 $\times$ His-WW-I–IV and GST-C-tail was as described above.

Ubiquitination Assay

For detection of ubiquitinated CXCR4, HEK293 cells were transiently transfected using FuGENE-6 transfection reagent with DNA encoding HA-tagged CXCR4 and FLAG-tagged ubiquitin. Cells were treated with CXCL12 (100 nM) for 30 min followed by immunoprecipitation of CXCR4 and immunoblotting to detect incorporated ubiquitin, essentially as described previously (Marchese and Benovic, 2001; Marchese *et al.*, 2003).

Fluorescence Resonance Energy Transfer (FRET) Experiments

Fluorescence imaging was performed using an inverted microscope equipped with a 1.49 numerical aperture (NA) objective, and a back-thinned camera (iXon 887; Andor Technology, Belfast, Northern Ireland). The detector was cooled to -100°C , by using a recirculating liquid coolant system (Koolance, Auburn, WA). Image acquisition and acceptor photobleaching were automated with custom software macros in MetaMorph (Molecular Devices, Sunnyvale, CA) that controlled motorized excitation/emission filter wheels (Sutter Instrument, Novato, CA) with filters for CFP and YFP (Semrock, Rochester, NY). HEK293 cells transiently transfected with cDNAs encoding HA-CXCR4-YFP and AIP4-CFP were plated onto poly-L-lysine-coated chambered borosilicate coverglass (Nalge Nunc International). The cells were

serum starved for 1 h before measuring the FRET between HA-CXCR4-YFP and AIP4-CFP, and they were maintained in the same serum-free medium throughout the course of the experiment. FRET between HA-CXCR4-YFP and AIP4-CFP was measured both in the absence and presence of 100 nM CXCL12 at 20–23°C by using the acceptor photobleaching method (Kenworthy, 2001; Kelly *et al.*, 2008). For measuring FRET in the presence of CXCL12, acceptor photobleaching was started 10 min after the manual addition of CXCL12. The progressive photobleaching protocol was as follows: 100-ms acquisition of CFP image, 40-ms acquisition of YFP image, followed by a 10-s exposure to selectively photobleach YFP (504/12-nm excitation). The apparent FRET efficiency was calculated from the fluorescence intensity of the CFP (427/10-nm excitation) donor before and after acceptor-selective photobleaching, according to the formula $\%FRET = [1 - (F_{\text{prebleach}}/F_{\text{postbleach}})] \times 100$.

Coimmunoprecipitation Assay

HEK293 cells transiently coexpressing FLAG-AIP4 and HA-CXCR4 or pcDNA3.0 were serum starved for 3–4 h and stimulated with either vehicle (DMEM + 0.5% FBS) or 30 nM CXCL12 for 15 min. Cells were washed once with ice-cold phosphate-buffered saline (PBS) and lysed in ice-cold buffer (20 mM Tris-Cl, pH 7.5, 150 mM NaCl, 1% Triton X-100, protease inhibitors [10 µg/ml each pepstatin, leupeptin, and aprotinin], and phosphatase inhibitor cocktails I and II [Sigma-Aldrich]). Cell lysates were sonicated, clarified by centrifugation at $21,000 \times g$ for 20 min at 4°C, and CXCR4 was immunoprecipitated using a polyclonal anti-HA antibody. Immunoprecipitates were analyzed for the presence of FLAG-AIP4 by SDS-PAGE followed by immunoblotting.

Total Internal Reflection Fluorescence (TIRF) Microscopy Experiments

HEK293 cells transiently coexpressing HA-CXCR4 wild-type, S324/5A, or S324/3D and AIP4-CFP were passaged onto poly-L-lysine (0.1 mg/ml)-coated coverglass chambers (Nalge Nunc International). The next day, cells were serum starved with DMEM containing 20 mM HEPES for at least 1 h before starting the experiment and maintained in the same medium at 20–23°C throughout the course of the experiment. TIRF imaging was performed with a Nikon TE2000-U inverted microscope. Illumination from a 449-nm diode laser (RGLase) was introduced through a Nikon TIRF II illuminator, reflected with a multiple band dichroic mirror (Chroma Technology, Rockingham, VT), and directed to the sample using a 1.49 NA objective. Images were obtained using a filter (Semrock) for CFP (472/30 nm), a computer-controlled emission filter wheel (Sutter Instrument), and an electron-multiplying-charge-coupled device camera (iXon 887; Andor Technology) cooled to –100°C with a recirculating liquid coolant system (Koolance). Image acquisition was automated with custom software macros in MetaMorph (Molecular Devices). Time-lapse images were obtained every 5 s for 10 min. After the first 15 images, the acquisition was paused briefly and resumed after addition of 100 nM CXCL12 to the cell chamber. Images were analyzed and processed using ImageJ 1.3v software (National Institutes of Health, Bethesda, MD). To calculate the area of the basal plasma membrane in contact with the coverslip, TIRF images were autothresholded and analyzed with the ImageJ macro “Analyze particles.” The image stack was then manually thresholded to select puncta, and particles of size 0.05–1.0 and circularity 0.5–1.0 were counted with the Analyze particles macro. This analysis yielded the mean density (puncta per square micrometer) before and after CXCL12 stimulation.

Determination of CXCR4 Phosphorylation by Confocal Immunofluorescence Microscopy by Using a Custom Phosphoserine-specific Antibody

A custom mAb that recognizes dually phosphorylated serine residues 324 and 325 was obtained from A and G Pharmaceutical (Baltimore, MD). The antibody was raised against the following keyhole limpet hemocyanin-conjugated peptide: SRG-pS-pS-LKILSKGKR (pSer324-pSer325). Supernatants from 28 hybridomas were initially screened against bovine serum albumin (BSA)-conjugated phosphorylated and unphosphorylated peptides by enzyme-linked immunosorbent assay (Supplemental Figure S3). Subsequently, supernatants from 17 hybridomas were used to screen HEK293 cells expressing HA-tagged CXCR4 and S324/5A by Western blotting (with dilutions up to 1:10), immunoprecipitation and confocal immunofluorescence microscopy. Although the data obtained by Western blotting and immunoprecipitation were inconclusive, one clone (5E11), which recognizes phospho-peptide S324/5 but not unphosphorylated peptide as determined by enzyme-linked immunosorbent assay, was found to recognize doubly phosphorylated CXCR4 on serine residues 324 and 325 by confocal immunofluorescence microscopy. HEK293 cells transfected with wild-type HA-CXCR4-YFP or S324/5A mutant were passaged onto poly-L-lysine-coated coverslips. After 48 h, cells were serum starved for 3–4 h in DMEM containing 20 mM HEPES, pH 7.4, followed by replacement with the same medium containing either vehicle (0.1% BSA in PBS) or ligand (30 nM CXCL12) for 5, 15, or 30 min. Cells were then washed with PBS, fixed with 3.7% formaldehyde-PBS solution for 15 min, and permeabilized with 0.01% saponin-PBS for 10 min at room temperature. Cells were incubated with 5% FBS in 0.01% saponin-PBS for 30

min at 37°C followed by immunostaining with clone 5E11 (hybridoma supernatant, 1:10 dilution) at 37°C for 30 min. Cells were washed and incubated with Alexa-Fluor 563-conjugated secondary antibody at 37°C for 30 min. Finally, cells were washed and fixed with 3.7% formaldehyde-PBS before mounting onto glass slides using VECTASHIELD mounting medium.

For CIP and λ phosphatase treatments, cells were fixed and permeabilized followed by incubation with either 200 U of CIP (New England Biolabs) at 37°C or 400 U of λ phosphatase (Sigma-Aldrich) at 30°C for 1 h. Control cells were incubated under similar conditions in the respective buffers only. Cells were washed with PBS and subject to blocking and subsequent staining and mounting steps as described above. Images were acquired using an LSM-510 laser scanning confocal imaging system (equipped with a 1.4-megapixel cooled extended spectral range RGB digital camera; Carl Zeiss, Thornwood, NY) with a C-Apo 40 $\times$ /1.2 water objective at 512 $\times$ 512 resolution. Acquired images were analyzed using ImageJ 1.3v software.

Degradation Assay

Agonist promoted degradation of HA-CXCR4 WT and the serine mutants was detected by immunoblot analysis, as described previously (Marchese *et al.*, 2003).

Statistical Analysis

One-way analysis of variance (ANOVA) and Student's *t* test were performed using GraphPad Prism version 4.00 for Macintosh (GraphPad Software, San Diego CA; www.graphpad.com). Two-way ANOVA was performed using GraphPad Prism and GB-STAT (Dynamic Microsystems, Silver Spring, MD).

RESULTS

AIP4 Binds Directly to CXCR4

The machinery responsible for ubiquitinating GPCRs remains poorly defined. We have shown that the E3 ubiquitin ligase AIP4 mediates agonist-promoted ubiquitination of the chemokine receptor CXCR4 at the plasma membrane and functions in endosomal sorting to target the receptor for lysosomal degradation (Marchese *et al.*, 2003). Although AIP4 colocalizes with CXCR4 at the plasma membrane upon agonist treatment, the determinants targeting AIP4 to CXCR4 remain unknown (Marchese *et al.*, 2003). Because lysine residues within the C-tail of CXCR4 are the sites for ubiquitin modification, we first assessed whether AIP4 interacted with the C-tail of CXCR4. Whole cell lysates prepared from HEK293 cells expressing FLAG-tagged AIP4 were incubated with the C-tail of CXCR4 fused to GST (Figure 1A) immobilized on glutathione-Sepharose resin, and bound AIP4 was detected by immunoblotting. As shown in Figure 1B, AIP4 bound to GST-C-tail but not to GST alone, suggesting that AIP4 interacts with the C-tail of CXCR4. To determine whether the interaction is direct or mediated by an AIP4-associated protein, we examined the binding of purified HIS-tagged full-length AIP4 to GST-C-tail. As shown in Figure 1C, purified HIS-AIP4 showed a substantial amount of binding to GST-C-tail, compared with GST alone, indicating that the interaction between CXCR4 and AIP4 is direct.

We next wanted to determine whether CXCR4 and AIP4 interact in cells upon agonist activation by coimmunoprecipitation. HEK293 cells transiently expressing HA-CXCR4 and FLAG-AIP4 were treated with vehicle or 30 nM CXCL12 for 15 min followed by immunoprecipitation of HA-CXCR4 and immunoblotting to determine the presence of FLAG-AIP4 in the immunoprecipitates. As shown in Figure 2A, coimmunoprecipitation of AIP4 with CXCR4 was enhanced upon agonist treatment compared with vehicle-treated cells, indicating that CXCL12 treatment promoted the interaction between CXCR4 and AIP4. To further characterize the interaction, we used FRET coupled with fluorescence microscopy, which are powerful techniques to measure protein-protein interactions in live cells (Piston and Kremers, 2007). To this end, CXCR4 and AIP4 were tagged with fluorescent proteins YFP and CFP, respectively, which when in proxim-

Figure 1. The carboxy-terminal tail of CXCR4 interacts with AIP4. (A) Schematic representation of the carboxy-terminal tail of CXCR4 fused to GST. CXCR4 is a seven transmembrane domain spanning integral plasma membrane protein. The predicted cytoplasmic carboxy-terminal tail (amino acid residues 308-352) was fused to GST to create GST-C-tail. (B) The C-tail of CXCR4 interacts with AIP4. Whole cell lysates from HEK293 cells expressing FLAG-tagged AIP4 were incubated with GST-C-tail (57 nM) or GST (71 nM) alone. Input represents 0.4% of the lysate used in the binding reactions. Immunoblot was probed with the anti-FLAG M2 antibody to detect FLAG-AIP4. Blot was stained with Ponceau-S to determine the levels of the GST proteins used in the binding assay. Data from one of three independent experiments are shown. (C) The C-tail of CXCR4 binds directly to AIP4. Histidine-tagged AIP4 (HIS-AIP4; ~5.2 pmol) was incubated with GST-C-tail (57 nM) or GST (71 nM) alone. Input represents ~0.2% of the purified HIS-tagged AIP4 used in the binding assay. Immunoblot was probed with an anti-HIS antibody to detect HIS-AIP4. A GelCode blue stained gel indicating the levels of the GST proteins used in the binding assay is shown. Data from one of three independent experiments are shown.

ity can undergo FRET (Piston and Kremers, 2007). YFP was placed at the carboxy terminus of HA-CXCR4, which does not interfere with its internalization and trafficking to lysosomes upon agonist stimulation (Bhandari *et al.*, 2007). AIP4 was tagged with CFP at its carboxy terminus, which does not seem to interfere with its activity (Supplemental Figure

1). To measure FRET between HA-CXCR4-YFP and AIP4-CFP, we followed an acceptor-selective photobleaching protocol as described previously (Kelly *et al.*, 2008). HEK293 cells were transiently transfected with HA-CXCR4-YFP and AIP4-CFP and FRET was monitored in the presence or absence of 100 nM CXCL12. Images from a representative cell treated with CXCL12 showing prebleach and postbleach YFP and CFP fluorescence are shown in Figure 2B. YFP fluorescence was selectively photobleached (Figure 2B, bottom), which led to enhanced CFP fluorescence, compared with prebleach fluorescence, indicating FRET between HA-CXCR4-YFP and AIP4-CFP (Figure 2B, top). The increased CFP fluorescence was observed at the cell periphery and in a perinuclear intracellular pool, reflecting the plasma membrane and an endosomal pool, compartments in which we have shown previously that CXCR4 and AIP4 colocalize (Marchese *et al.*, 2003). Figure 2C shows the time-dependent change in CFP emission intensity from cells treated with ($n = 17$) or without ($n = 20$) CXCL12, while undergoing progressive and complete photobleaching of YFP (data not shown). The AIP4-CFP emission significantly increased upon treatment of cells with CXCL12 compared with cells that were not treated with CXCL12 (unpaired Student's *t* test, $p < 0.0001$), suggesting an enhanced association between HA-CXCR4-YFP and AIP4-CFP compared with untreated cells. Therefore taken together, our data indicate that agonist activation promotes the interaction between CXCR4 and AIP4.

AIP4 WW Domains Are Critical for Binding to CXCR4

To further understand the mechanism of the interaction between AIP4 and CXCR4, we next sought to identify the determinants responsible for the direct binding between AIP4 and CXCR4. Although the WW domains of AIP4 have been shown to interact with proline-rich (i.e., PY) motifs within target proteins (Winberg *et al.*, 2000; Ingham *et al.*, 2005a), there is some suggestion that they may interact with noncanonical motifs (Ingham *et al.*, 2005a). Therefore, although the C-tail of CXCR4 does not contain proline residues, to understand the molecular basis for the interaction between CXCR4 and AIP4, we initially examined whether the WW domains of AIP4 mediate the interaction with CXCR4. Full-length AIP4 (GST-AIP4) and the four tandemly linked WW domains (GST-WW-I-IV) were expressed as GST fusion proteins (Figure 3A), purified on glutathione-Sepharose resin, and incubated with whole cell lysates prepared from cells expressing HA-CXCR4 and bound receptor was detected by immunoblotting. As shown in Figure 3B, CXCR4 bound to full-length GST-AIP4 as expected; and surprisingly, CXCR4 also bound to GST-WW-I-IV. To determine whether other regions of AIP4 could also bind to CXCR4, we created a GST-fusion protein in which the WW domains were deleted (GST-AIP4 Δ WW) and tested the ability of this mutant to bind to CXCR4. As shown in Figure 3C, CXCR4 binding to GST-AIP4 Δ WW was significantly diminished compared with GST-AIP4 and GST-WW-I-IV, suggesting that the WW domains represent the main CXCR4 binding region. To confirm that the WW domain mediated interaction was not via an intermediate protein, we next performed the binding experiments using purified proteins. As shown in Figure 3D, GST-C-tail bound to increasing amounts of purified HIS-tagged WW-I-IV, indicating a direct interaction between the C-tail of CXCR4 and the WW domains of AIP4. Thus, our data reveal that AIP4 WW domains interact directly with the C-tail of CXCR4, likely through a novel noncanonical recognition sequence.

Figure 2. Agonist mediated interaction between CXCR4 and AIP4. (A) Agonist enhances the interaction between CXCR4 and AIP4. Serum-starved HEK293 cells cotransfected with FLAG-AIP4 and HA-CXCR4 or pcDNA3.0 were treated with either vehicle (DMEM containing 0.5% FBS) or CXCL12 (30 nM) for 15 min. The clarified whole cell lysates (WCL) were immunoprecipitated (IP) by using an anti-HA antibody and analyzed by SDS-PAGE and immunoblotting to detect bound FLAG-AIP4. Immunoprecipitates were also immunoblotted (IB) for HA-CXCR4 and WCL were immunoblotted to detect the expression of FLAG-AIP4 and HA-CXCR4. The HA antibody in the CXCR4 IP recognizes multiple bands, which are likely differentially glycosylated forms of the receptor. Immunoblots were stripped and reprobed for actin to assess loading. The bar graph represents average FLAG-AIP4 binding normalized to the level of receptor in the IPs. Error bars represent the SEM from three independent experiments. Binding of FLAG-AIP4 to CXCR4 in the presence of CXCL12 was significantly increased over its binding to the unstimulated receptor. * $p < 0.05$, unpaired t test. (B) FRET analysis of fluorescent protein-tagged CXCR4 and AIP4 in live cells. Shown are images of a representative cell treated with CXCL12. Prebleach and postbleach donor (HA-CXCR4-YFP; yellow) and acceptor (AIP4-CFP; cyan) images are shown. Arrows indicate cell periphery where FRET was observed. (C) In total, 20 cells without CXCL12 (–) and 17 cells with CXCL12 (+) treatment were analyzed from three independent experiments. The average F/F_0 ratio for AIP4-CFP, where F_0 and F are the donor emissions before and after progressive photobleaching, respectively, is plotted against time. Fluorescence intensity was measured using the whole cell for the analysis. The error bars represent the SE. The data were subject to an unpaired t test ($p < 0.0001$).

WW domains are characterized by the presence of two conserved tryptophan residues and are thought to adopt a similar structure; however, their recognition sequence preferences may differ (Zarrinpar and Lim, 2000). Therefore, to gain further mechanistic insight into the interaction, we wanted to determine the ability of each of the four individual WW domains of AIP4 to interact with CXCR4. The four WW domains were expressed individually as GST-fusion proteins and assessed for their ability to bind HA-CXCR4 expressed in HEK293 cells. As shown in Figure 4A, CXCR4 bound to GST-WW-I and GST-WW-II, but not to GST-WW-III and GST-WW-IV, indicating that the interaction with CXCR4 is specific to WW domains I and II. Interestingly, binding to individual WW domains I and II was not as great as that observed with binding to WW-I-IV or full-length AIP4, suggesting higher avidity of binding when the WW domains are tandemly linked, and consistent with the pos-

sibility that AIP4 may bind to two CXCR4 molecules through simultaneous interactions via both WW domains I and II. Regardless, our data indicate that CXCR4 has a preference for WW domains I and II, via a novel noncanonical recognition sequence.

To characterize the interaction further, we next wanted to identify the residues within WW domains I and II that are important for the interaction. Typically, the second conserved tryptophan residue within WW domains forms part of the binding pocket that enables interactions with proline-based recognition sequences of target proteins (Zarrinpar and Lim, 2000). For AIP4 WW domains I and II, the second conserved tryptophan residue corresponds to Trp313 and Trp345, respectively (Figure 4B). To assess whether these residues are also involved in the interaction with CXCR4, we mutated Trp313 and Trp345 to alanine either together or alone within the context of GST-WW-I-IV and then assessed

Figure 3. The WW domains of AIP4 mediate the interaction with CXCR4. (A) Schematic representation of AIP4. AIP4 has a C2 domain, a PRR, four tandemly linked WW domains, and a catalytic HECT-domain. GST was fused to the amino terminus of full-length AIP4 (GST-AIP4), to AIP4 lacking the WW domains (GST-AIP4ΔWW) and to the WW domains alone (GST-WW-I-IV). (B and C) CXCR4 interacts with the WW domains of AIP4. (B) Whole cell lysates (WCL) from HEK293 cells transiently expressing HA-CXCR4 and empty vector (pcDNA3) were incubated with 1 μ g of GST-AIP4, GST-AIP4ΔWW, GST-WW-I-IV, or GST alone. (C) WCL from HEK293 cells stably expressing HA-CXCR4 were incubated with equimolar amounts (~22 pM) of GST-AIP4, GST-AIP4ΔWW, GST-WW-I-IV, or GST alone. Samples were resolved by SDS-PAGE, followed by immunoblotting with an anti-HA antibody (top) to detect bound HA-CXCR4. Coomassie- (B) and GelCode blue (C)-stained gels (bottom) indicate the level of the GST fusion proteins used in the binding experiments. Input represents ~0.6% (B) and 0.2% (C) of the lysate that was used for the binding experiment. Data from one of three experiments are shown. Receptor levels were determined by densitometry and average receptor binding was expressed as the percentage of control wild-type AIP4 binding (WT) $\pm$ SEM from three independent experiments (C, right). Please note that the SEM for Δ WW binding is $\pm$ 0.39. Data were analyzed by a one-way ANOVA, followed by Bonferroni's post hoc test. Binding to the Δ WW mutant was significantly different from WT and WW-I-IV binding. * p < 0.05. (D) The C-tail interacts directly with AIP4 WW domains. Increasing amounts of HIS-tagged WW-I-IV were incubated with equal amounts of GST-C-tail or GST alone. Bound HIS-WW-I-IV (60% of sample) was detected by immunoblotting with a HIS antibody conjugated to HRP. A known amount of HIS-WW-I-IV (3 ng) was also immunoblotted. The asterisk represents nonspecific binding of the HIS-HRP antibody to GST-C-tail. HIS-WW-I-IV binding was determined by densitometric analysis and normalization to GST-C-tail levels. Data are the mean HIS-WW-I-IV binding $\pm$ SE from four experiments. The data were analyzed by a one-way ANOVA followed by Bonferroni's post hoc test; p < 0.05 between 100 and 500 ng.

Figure 4. Identification of residues within WW domains I and II that are critical for binding to CXCR4. (A) CXCR4 interacts with AIP4 WW domains I and II. Whole cell lysates (WCL) from HEK293 cells expressing HA-CXCR4 were incubated with GST alone (~110 nM), GST-AIP4 (~25 nM), GST-WW-I-IV (~55 nM) or GST fusions of each of the four individual WW domains (~100 nM). Immunoblot was probed with an anti-HA antibody to detect bound HA-CXCR4. A Coomassie-stained gel is shown to indicate the level of GST proteins used in the binding assay. Input represents 1.5% of the lysate used in the binding assay. Shown are data from one of three independent experiments. (B) Alignment of the WW domains from AIP4 and several members of the Nedd4-like family of E3 ligases. The four WW domains of AIP4 are shown aligned. Residues that are either identical or conserved to both WW domains I and II are high-lighted black. Residues that are unique to each of the four WW domains are boxed and shaded gray. Sequence of the Pin1 WW domain is also shown and the critical arginine residue shown to be important for phosphorylation mediated binding to Pin1 is shaded. The WW domains from other members of the Nedd4-like HECT domain family are shown (WWP1, WWP2, Nedd4, and Rsp5). The conserved tryptophan residues from which the WW domain derives its name are boxed. A space (-) has been inserted to maximize the alignment. The three conserved β sheets that are connected by two loops are indicated above the sequence. Residues are numbered relative to their appearance in the full-length protein. The single-letter amino acid code is used. Sequences were obtained from the GenBank flat files under the following accession numbers: NP_113671 (AIP4), AAC51324 (WWP1), AAC51325 (WWP2), NP_011051 (yeast RSP5), P46934 (hNedd4), and AAC50492 (Pin1). (C–E) Identification of critical residues within WW domains I and II important for binding to CXCR4. WCL from HEK293 cells expressing HA-CXCR4 were incubated with the indicated GST-WW domain fusion protein (~25–50 nM) or GST (~45 nM) alone. The immunoblot (IB) was probed with an anti-HA antibody to detect bound receptor (top). IB was stripped and reprobed with an anti-GST antibody (C and E) or gel was stained with GelCode blue (D) to reveal level of GST fusion proteins used in the assay. Input represents 0.3% of the lysate used in the binding assay. Bound receptor levels were determined by densitometry and average receptor binding was expressed as the percentage of control GST-WW-I-IV wild-type (WT) binding $\pm$ SEM from three (D and E) or 5 (C) independent experiments. Data were analyzed by either a *t* test (E) or a one-way ANOVA and Bonferroni's post hoc test (C and D). Binding of the double mutants (W313/W345A and Q297/N329A) and the quadruple mutant (W313/Q297/W345/N329A) to CXCR4 was significantly different from wild-type binding. C, **p* < 0.01; D, **p* < 0.05; and E, **p* < 0.01. (F) Purified HIS-tagged WWI-IV WT, Q297/N329A, W313/W345A, and 4A (~70 nM) were incubated with equimolar amounts of either GST-C-tail WT or GST alone. Immunoblot was probed with an anti-HIS antibody to detect HIS-WWI-IV. The input represents ~2% of the purified His-WWI-IV (WT or mutants) used in the binding assay. The immunoblot was stripped and reprobed with an anti-GST antibody to detect the levels of GST proteins used in the binding assay. HIS-WWI-IV binding was determined by densitometric analysis, and data represent the mean $\pm$ SE from three independent experiments. Data were analyzed by a one-way ANOVA and Bonferroni's post hoc test. The binding of HIS-WWI-IV mutants to GST-C-tail was significantly different from that of wild-type (WT). **p* < 0.01.

the ability of the GST-fusion proteins to bind to HA-tagged CXCR4 expressed in HEK293 cells. As shown in Figure 4C,

the extent of HA-CXCR4 binding to GST-WW-I-IV containing individual point mutations (W313A or W345A) was

Figure 4. (Continued).

somewhat diminished compared with wild-type GST-WW-I-IV binding. However, binding of HA-CXCR4 to the double mutant (W313A/W345A) was significantly reduced by ~60% compared with wild-type binding. Together, these data indicate that conserved residues Trp313 and Trp345 within WW domains I and II, respectively, are important for CXCR4 recognition, although the mechanism for the interaction remains to be determined.

Because we still observed significant binding to the double tryptophan mutant, it is likely that other residues within the WW domains contribute to the interaction with CXCR4. Multiple residues are known to be important for standard WW domain and proline-based interactions (Verdecia *et al.*, 2000; Kasanov *et al.*, 2001). To identify such residues, we aligned the amino acid sequence of the four AIP4 WW domains to each other to determine whether we could iden-

tify amino acid residues unique to WW domains I and II, but not III and IV, because we reasoned that these residues were likely to be important for the specificity in binding of the WW domains (Figure 4B). Using this strategy, we identified two conserved residues, Gln297 and Asn329, in the loop region that connects the first β sheet with the second β sheet of WW domains I and II, respectively (see Figure 4B for sequence). Interestingly, these residues are located at the analogous position to an arginine residue that has been shown to be important for binding of group IV WW domains to phospho-serine/threonine-Pro [p(Ser/Thr)] motifs (Verdecia *et al.*, 2000). To determine whether Gln297 and Asn329 are important for binding to CXCR4, we changed them to alanine residues within the context of GST-WW-I-IV either together or alone and tested the ability of these fusion proteins to bind to HA-tagged CXCR4 expressed in HEK293

cells. As shown in Figure 4D, binding of HA-CXCR4 to the single GST-WW-I-IV point-mutants (Q297A and N329A) was similar to that observed with wild-type GST-WW-I-IV. However, binding of HA-CXCR4 to the double mutant (Q297A/N329A) was significantly reduced (by ~58%; $p < 0.05$) compared with its binding to wild-type GST-WW-I-IV, suggesting that Gln297 and Asn329 are important for binding HA-CXCR4. We next determined the effect of combining

the tryptophan mutations with the glutamine and asparagine mutations (W313/W345/Q297/N329A or 4A) on the binding of WWI-IV to CXCR4. As shown in Figure 4E, when all four residues were simultaneously changed to alanine residues, binding to CXCR4 was almost abolished, suggesting that these residues are important for mediating the interaction with CXCR4. To ensure that these WW domain residues are indeed mediating the direct interaction with the

Figure 5. CXCR4 C-tail serine residues mediate the interaction with AIP4. (A) CXCR4 C-tail amino acid sequence. Shown is the amino acid sequence of the carboxy-terminal tail of CXCR4 and the various serine receptor mutants. The single-letter amino acid code is used. (B and C) CXCR4 serine receptor mutant binding to AIP4. (B) Whole cell lysates (WCL) from HEK293 cells expressing wild-type HA-CXCR4 and the indicated serine mutants were incubated with GST-WW-I-IV (~17 nM) or GST (~97 nM) alone. Bound receptor was detected by immunoblotting (top). Blots were stripped and reprobed with an anti-GST antibody (bottom). Input represents 0.3% of the lysate used in the binding assay. Shown are representative blots. (C) Bound receptor levels were determined by densitometry and average receptor binding was expressed as the percentage of control CXCR4 binding $\pm$ SEM from five independent experiments. Data were analyzed by a Kruskal–Wallis one-way ANOVA, followed by Dunn’s post hoc test. Binding to S324/5A was significantly different from WT and S330A binding. * $p < 0.05$. (D) FRET analysis between S324/5A and AIP4. Cells expressing HA-S324/5A-YFP and AIP4-CFP were treated and the FRET efficiency was calculated as described in *Materials and Methods*. Shown is the average FRET efficiency between HA-S324/5A-YFP and AIP4-CFP from cells treated with (n = 23) or without (n = 26) CXCL12. Data from Figure 2 were used to calculate the FRET efficiency between HA-CXCR4-YFP and AIP4-CFP. Data were analyzed by a two-way ANOVA ($p < 0.01$), followed by a Bonferroni’s post hoc test (* $p < 0.0001$, between CXCL12 treated wild-type and S324/5A cells).

tail of CXCR4 we further examined the interaction using purified proteins. As shown in Figure 4F, binding of GST-C-tail to HIS-WW-I-IV double mutants (Q297A/N329A and W313A/W345A) and the quadruple mutant (4A) was significantly ($p < 0.01$) attenuated compared with wild-type HIS-WW-I-IV, further indicating that the C-tail of CXCR4 interacts directly with AIP4 WW domains involving residues Q297, N329, W313, and W345.

CXCR4 C-Tail Serine Residues Mediate the Interaction with AIP4

The C-tail of CXCR4 seems to be the major site for AIP4 binding and ubiquitination, however the critical residues that mediate this interaction are not known. Interestingly, in previous studies we have shown that CXCR4 C-tail serine residues 324, 325, and 330 are important for agonist-promoted receptor degradation (Marchese and Benovic, 2001). However, the mechanistic basis for their role in CXCR4 degradation remains unknown. One possibility is that these serine residues are important for mediating AIP4 binding and thus subsequent ubiquitination and degradation of the receptor. To this end, we first examined whether CXCR4 C-tail serine residues mediate binding to AIP4 by testing the ability of GST-WW-I-IV to bind to CXCR4 serine mutant receptors S324/5A and S330A (Figure 5A). Whole cell lysates prepared from HEK293 cells expressing HA-tagged wild-type CXCR4 or C-tail mutants were incubated with GST-WW-I-IV or GST alone and bound receptors were detected by immunoblotting. As shown in Figure 5, B and C, GST-WW-I-IV binding to the S324/5A CXCR4 mutant was significantly reduced by ~67% compared with its binding to wild-type receptor. In contrast, binding to S330A mutant was similar to wild-type receptor binding, suggesting that this serine residue is not involved in AIP4 binding. These findings suggest that serines residues S324/5 within the C-tail of CXCR4 are important for binding to the WW domains of AIP4.

To determine the importance of these serine residues in mediating an interaction with AIP4 in live cells, we examined the interaction between S324/5A and AIP4 via FRET analysis. HEK293 cells transiently transfected with HA-S324/5A-YFP and AIP4-CFP were either treated or not treated with CXCL12 and FRET was monitored using the acceptor photobleaching protocol described above. The average FRET efficiency between HA-S324/5A and AIP4-CFP in the absence of CXCL12 treatment was 9.3% ($n = 26$), which was similar to the average FRET efficiency in the presence of CXCL12 treatment (10.8%; $n = 23$), suggesting that agonist fails to enhance the interaction between S324/5A and AIP4 (Figure 5D). This is in contrast to what we observed with wild-type CXCR4. We calculated the FRET efficiency from the data in Figure 2, in which we examined the interaction between HA-CXCR4-YFP and AIP4-CFP. In this case, the average FRET efficiency was 10.3% in the absence of CXCL12, which increased significantly ($p < 0.001$) to 16.7% in the presence of CXCL12, indicating that agonist enhances the interaction between CXCR4 and AIP4 (Figure 5D). Interestingly, FRET also occurred in the absence of agonist, suggesting that CXCR4 and AIP4 interact constitutively. We also observed similar amounts of FRET under basal conditions with S324/5A compared with wild-type CXCR4 (9.3 vs. 10.3% for S324/5A and wild-type CXCR4, respectively). This is in contrast to the GST-WW-I-IV interaction studies in which there was a decreased level of binding to S324/5A compared with wild-type receptor, even though these experiments were performed in the absence of agonist treatment (Figure 5B). It is

possible that coexpression of fluorescent protein-tagged receptors and AIP4 contributes to a high degree of constitutive association that is not dependent on serine residues 324 and 325. Nevertheless, upon treatment with agonist, a significant increase in the FRET efficiency (~62%) was observed only between wild-type receptor and AIP4 and not between the mutant receptor and AIP4, suggesting that serine residues 324 and 325 are critical for mediating the agonist promoted interaction between CXCR4 and AIP4 in cells.

We have previously shown that AIP4 mediates agonist-promoted ubiquitination and degradation of CXCR4 (Marchese *et al.*, 2003) and that serine residues 324 and 325 are critical for mediating agonist promoted degradation of CXCR4 (Marchese and Benovic, 2001). To determine whether these serine residues are also important for CXCR4 ubiquitination, we assessed the ubiquitination status of the S324/5A mutant. HEK293 cells transfected with HA-tagged wild-type CXCR4 or S324/5A, plus FLAG-tagged ubiquitin

Figure 6. CXCR4 C-tail serine residues 324 and 325 are important for CXCR4 ubiquitination and degradation. (A) Ubiquitination of S324/5A is attenuated compared with wild-type CXCR4. HEK293 cells were transfected with the indicated CXCR4 constructs and FLAG-tagged ubiquitin. Cells were treated in the presence or absence of 100 nM SDF for 30 min at 37°C, followed by immunoprecipitation (IP) of the receptor and immunoblotting (IB) to detect the incorporation of epitope-tagged ubiquitin. Blots were then stripped and reprobed with an anti-HA mAb to assess receptor levels. Shown are representative blots from one of six independent experiments. Ubiquitinated CXCR4 levels were assessed by densitometric analysis and the -fold increase upon CXCL12 treatment compared with vehicle treatment was normalized to receptor levels present in the immunoprecipitates. Ubiquitination of CXCR4 was significantly increased compared with the S324/5A mutant. Unpaired t test: $*p < 0.03$. (B) Involvement of serine residues 324 and 325 in agonist-promoted degradation of CXCR4. HEK293 cells transfected with the indicated HA-tagged CXCR4 constructs were treated in the presence or absence of 10 nM SDF for 3 h at 37°C in DMEM containing 10% FBS and 50 μ g/ml cycloheximide, essentially as described previously (Marchese and Benovic, 2001). Equal amounts of lysates were subject to SDS-PAGE and immunoblotting to detect receptor levels. Receptor levels were assessed by densitometric analysis and normalized to tubulin levels. The bars represent the average percent receptor degraded in cells treated with agonist compared with vehicle-treated cells from five independent experiments. Error bars represent the SE of the mean.

were treated with agonist for 30 min followed by receptor immunoprecipitation and immunoblotting to detect incorporation of ubiquitin, essentially as described previously (Marchese and Benovic, 2001). As shown in Figure 6A, agonist treatment failed to promote ubiquitination of the S324/5A mutant compared with wild-type CXCR4, which is

consistent with the inability of this mutant to interact with AIP4 and undergo agonist promoted degradation (Figure 6B; Marchese and Benovic, 2001). To determine the relative contribution of serine residues 324 and 325 on agonist-promoted degradation of CXCR4, we examined the ability of the individual S324A and S325A mutants to undergo agonist

Figure 7. CXCR4 phospho-mimetic mutants show enhanced binding to AIP4. (A) The amino acid sequence of the carboxy-terminal tail of CXCR4 and the various serine-to-aspartic acid receptor mutants. (B) CXCR4 phospho-mimetic mutant binding to GST-WW-I-IV. Whole cell lysates from HEK293 cells expressing wild-type HA-CXCR4 or the indicated serine mutants were incubated with GST-WW-I-IV (~12 nM) or GST (~38 nM) alone. Immunoblot was probed with an anti-HA antibody to detect bound receptor. Shown is an immunoblot to indicate the level of the GST proteins used in the binding assay. Input represents 0.3% of lysate used in the binding assay. Receptor levels were determined by densitometry and average receptor binding was expressed as the percentage of control CXCR4 binding $\pm$ SEM from four independent experiments. (C) Binding analysis using purified proteins. Purified HIS-tagged WW-I-IV (~70 nM) was incubated with equimolar amounts of either the wild-type C-tail, S324/325A, and S324/325D fused to GST or GST alone. Immunoblot was probed with an anti-HIS antibody to detect bound HIS-WW-I-IV. The input represents ~1% of the purified His-WWI-IV used in the binding assay. The immunoblot was stripped and reprobed with an anti-GST antibody to detect the levels of GST proteins used in the binding assay. HIS-WWI-IV binding was determined by densitometric analysis, and data represent the mean $\pm$ SE from three independent experiments. (D) Enhanced agonist-promoted degradation of CXCR4 phospho-mimetic mutant S324/5D. Degradation of HA-tagged CXCR4 was assessed in cells treated with 10 nM SDF for 30 min at 37°C, essentially as described previously (Marchese and Benovic, 2001). The bars represent the average percentage of receptor degraded in cells treated with agonist compared with vehicle-treated cells from four independent experiments. Error bars represent the SE of the mean. Degradation of S324/5D was significantly enhanced as compared with wild-type receptor degradation (average mean, 3.8 vs. 27.2%). Unpaired *t* test, **p* < 0.03.

promoted degradation. As shown in Figure 6B, the degradation of the individual mutants (S324A and S325A) was modestly affected compared with the S324/5A double mutant, suggesting that both residues are needed for mediating agonist-promoted degradation of CXCR4. Thus, together our data suggest that the recruitment of AIP4 through its interaction with serine residues 324 and 325 within the C-tail of CXCR4 is critical for receptor ubiquitination and degradation.

CXCR4 Phosphorylation Is Important for AIP4 Binding

Because serine residues are potential sites of phosphorylation, we next wanted to investigate the role of CXCR4 phosphorylation in AIP4 recruitment. We generated phosphomimetic mutants of HA-CXCR4 by mutating serine residues 324 and 325 to aspartic acid (Figure 7A) and tested the ability of these receptor mutants expressed in HEK293 cells to bind to GST-WW-I-IV and GST alone. As shown in Figure 7B, binding of the individual aspartic acid mutants S324D and S325D and the double aspartic acid mutant S324/5D to GST-WW-I-IV was enhanced compared with wild-type CXCR4. Similarly, binding of purified HIS-WW-I-IV to GST-C-tail-S324/5D was enhanced, whereas binding to the S324/5A mutant was decreased, compared with the wild-type C-tail (Figure 7C). Consistent with the increased ability of the S324/5D mutant to bind to the WW domains of AIP4, degradation of S324/5D was significantly enhanced compared with wild-type receptor at an early time point (30

min) after agonist treatment (Figure 7D). Together, our data suggest that a negative charge located at positions 324 and 325 can promote the CXCR4/AIP4 interaction and receptor degradation.

We next investigated whether these serine residues are indeed phosphorylated upon receptor activation by generating a custom mAb (clone 5E11) that recognizes simultaneous phosphorylation of serine residues 324 and 325 (pS³²⁴pS³²⁵). Cells were treated with CXCL12 for 15 min and the phosphorylation status of CXCR4 was assessed by confocal immunofluorescence microscopy by using clone 5E11. As shown in Figure 8, A and B, CXCR4 was robustly phosphorylated on serine residues 324 and 325 upon treatment with CXCL12 for 15 min but not in vehicle-treated cells (0.1% BSA in PBS). Notably, 5E11 staining completely overlapped with YFP-tagged CXCR4 only at the cell surface and not on intracellular receptor (Figure 8B, yellow in merged panel), suggesting that these residues were simultaneously phosphorylated at the cell surface and dephosphorylated before or once CXCR4 appeared on endosomes. To confirm that 5E11 recognizes serine residues 324 and 325, we assessed the ability of 5E11 to immunostain the mutant S324/5A upon agonist treatment. As shown in Figure 8, C and D, 5E11 failed to recognize the S324/5A mutant expressed to similar levels as the wild-type receptor, confirming that serine residues 324 and 325 are part of the epitope recognized by 5E11. Also, of note, cells that did not express CXCR4 were not reactive to 5E11 (Figure 8B), further con-

Figure 8. CXCR4 C-tail serine residues 324 and 325 are phosphorylated upon agonist activation. HEK293 cells transiently expressing HA-tagged CXCR4-YFP (A, B, E, and F) or S324/5A-YFP (C and D) were incubated with either vehicle or CXCL12 for 15 min. Cells were fixed, permeabilized, and immunostained using an anti-phosphoserine 324/5 mAb (5E11). Images were captured under identical settings. To dephosphorylate receptors, permeabilized cells were treated with calf intestinal alkaline phosphatase (E) or λ phosphatase (F) before immunostaining with 5E11. HA-CXCR4-YFP- and 5E11-labeled CXCR4 images were pseudocolored as green and red, respectively, and merged using ImageJ software. Yellow in merged images represents S324/5 phosphorylated CXCR4. DIC images of the cells are shown (right). Representative images of cells analyzed from three independent experiments are shown. Bars, 10 μ m.

firming the specificity of this antibody toward CXCR4. To confirm that 5E11 recognizes phosphorylated CXCR4, we examined the staining pattern of 5E11 in cells expressing wild-type CXCR4 that were treated with agonist followed by incubation with λ phosphatase, alkaline phosphatase, or buffer alone. Under these conditions, 5E11 was nonimmunoreactive in cells treated with lambda phosphatase and alkaline phosphatase (Figure 8, E and F), but not in cells treated with buffer alone (data not shown), suggesting that CXCR4 was dephosphorylated and that 5E11 indeed recognizes phosphorylated CXCR4. Together, these data indicate that CXCR4 is simultaneously phosphorylated on serine residues 324 and 325 at the plasma membrane upon activation with CXCL12.

To investigate the time course of phosphorylation, we treated cells with CXCL12 for 5, 15, and 30 min and then assessed the phosphorylation status of CXCR4 on serine residues 324 and 325. We observed very little phosphorylation at 5 min (Figure 9, A and B), which peaked at 15 min (Figure 9, C and D) of agonist treatment, a time point at which CXCR4 is predominantly on the cell surface and which coincides with the time at which we observed an enhanced interaction between CXCR4 and AIP4 (Figure 2). We did not observe any immunostaining at 30 min after agonist treatment (Figure 9, E and F), at which point most of the receptor had been internalized, suggesting that the

serine residues 324 and 325 are dephosphorylated once the receptor appears on endosomes. Therefore, these data indicate that CXCR4 is simultaneously phosphorylated at the plasma membrane on serine residues 324 and 325 but is dephosphorylated before or upon internalization of the receptor onto endosomes. Together, these data suggest a mechanism whereby phosphorylation of serine residues 324 and 325 at the plasma membrane promotes recruitment of AIP4 directly to CXCR4.

We next set out to determine the role of phosphorylation of serine residues 324 and 325 on the localization of AIP4 to the plasma membrane. To do this, we performed TIRF microscopy on HEK293 cells transiently coexpressing AIP4-CFP and wild-type HA-CXCR4, S324/5A or the phosphomimetic mutant S324/5D. After serum starvation, cells were treated with or without CXCL12 and time lapse images were collected in TIRF mode to allow us to only focus on events occurring at or near the cell surface (evanescent field <500 nm). Figure 10A shows a representative cell from each transfection condition before and after agonist stimulation. In cells expressing wild-type CXCR4, we observed several AIP4-CFP puncta before stimulation, but their number increased upon agonist treatment (Figure 10A). Comparison of the puncta density (puncta per square micrometer) on the plasma membrane before and after agonist treatment revealed that agonist treatment promoted a significant in-

Figure 9. Time course of CXCR4 phosphorylation. HEK293 cells transiently expressing HA-CXCR4-YFP were incubated with vehicle or CXCL12 for 5 (A and B), 15 (C and D), or 30 (E and F) min. Cells were fixed, permeabilized, and immunostained with 5E11. HA-CXCR4-YFP- and 5E11-stained receptor images were pseudocolored as green and red, respectively, and merged using ImageJ software. Yellow in merged images represents S324/5 phosphorylated CXCR4. DIC images of the cells are also shown (right). Shown are representative images of cells analyzed from three independent experiments. Bars, 10 μ m.

Figure 10. AIP4 localizes to the plasma membrane upon activation of CXCR4. (A) Time-lapse images were acquired in TIRF mode for HEK293 cells transiently coexpressing AIP4-CFP and HA-CXCR4-WT, S324/5A, or S324/5D before and after treatment with 100 nM CXCL12 at $\sim 23^\circ\text{C}$. In total, 121 images were acquired over a period of 10 min. Shown are representative images of cells before and after treatment with agonist from each transfection. Puncta represent AIP4-CFP clusters at or near the cell surface attached to the glass support (depth of evanescent field < 500 nm). (B) The graph represents the average density of AIP4-CFP puncta within the evanescent field. The average puncta density was calculated from 15 time-lapse images per cell before agonist treatment from a total of eight, nine, and 10 cells examined from CXCR4, S324/5A, and S324/5D transfected cells, respectively, from three independent experiments. The data collected after agonist treatment represent the maximal average puncta density observed from 15 continuous time-lapse images per cell. Data were analyzed by a two-way ANOVA ($p < 0.01$), followed by a paired t test with a Bonferroni correction revealing a significant increase in puncta density after agonist treatment in cells expressing wild-type CXCR4 ($*p < 0.05$). Bar, 7 μm .

crease in puncta density of AIP4-CFP ($\sim 30\%$) in cells expressing wild-type CXCR4 (Figure 10B), suggesting that AIP4 is recruited to the plasma membrane upon agonist

activation of CXCR4. Cells expressing S324/5A, however, showed markedly lower AIP4-CFP puncta density in the absence of agonist compared with cells expressing wild-type receptor (Figures 10, A and B). In addition, agonist treatment failed to increase the puncta density, consistent with the idea that serine residues 324 and 325 are required for binding and recruitment of AIP4 to the receptor at the cell surface. In contrast, cells expressing the phosphomimetic mutant S324/5D without agonist treatment showed a puncta density that was similar to that of cells expressing wild-type receptor that were treated with agonist, suggesting that a negative charge imparted by aspartic acid residues 324 and 325 leads to enhanced binding and recruitment of AIP4 to the plasma membrane. Interestingly, puncta density did not change significantly after agonist treatment. Together, our data suggest that the agonist-dependent phosphorylation of serine residues 324 and 325 in the C-tail of CXCR4 is responsible for the recruitment of AIP4 to or in the vicinity of the plasma membrane.

DISCUSSION

The posttranslational modification of GPCRs with ubiquitin is important for endocytic trafficking (Marchese *et al.*, 2008). Although several GPCRs are modified with ubiquitin, the identities of the ubiquitin ligases and the mechanisms that control recruitment of the ubiquitination machinery to these receptors remain largely unknown. We showed previously that CXCR4 is modified with ubiquitin by the E3 ligase AIP4, which functions as an endosomal sorting signal to target CXCR4 to multivesicular bodies and its subsequent degradation (Marchese and Benovic, 2001; Marchese *et al.*, 2003). In this study, we have attempted to determine the mechanism by which AIP4 is targeted to CXCR4. Collectively, our data show that agonist activation can promote a direct interaction between CXCR4 and AIP4. To our knowledge, this represents the first description of a direct interaction between an E3 ubiquitin ligase and an activated GPCR. Unlike the $\beta_2\text{AR}$, which requires arrestin-3 for the recruitment of the E3 ligase Nedd4-1 to the receptor (Shenoy *et al.*, 2001, 2008), arrestins do not play a role in AIP4-mediated ubiquitination of CXCR4 (Bhandari *et al.*, 2007). Moreover, our studies reveal that the interaction occurs between CXCR4 C-tail serine residues and the WW domains of AIP4, suggesting a novel recognition motif for WW domains that to our knowledge has not been described previously in the literature.

The novel AIP4 recognition sequence on CXCR4 includes C-tail serine residues 324 and 325. Using a custom phospho-specific antibody, we show that CXCR4 is simultaneously phosphorylated on serine residues 324 and 325 at the plasma membrane. The time of maximal phosphorylation (~ 15 min) coincides with the time when AIP4 interacts with CXCR4 as assessed by coimmunoprecipitation and FRET analysis (Figure 2) and when we observe increased AIP4 puncta density near the plasma membrane by TIRF microscopy (Figure 10). These data are consistent with a mechanism whereby agonist-mediated phosphorylation of serine residues 324 and 325 promotes AIP4 recruitment and binding to the receptor at the cell surface for subsequent ubiquitination and degradation of CXCR4. Interestingly, both serine residues 324 and 325 are part of a previously characterized stretch of amino acids that mediates CXCR4 degradation (Marchese and Benovic, 2001). We have shown previously that agonist-promoted degradation of S324/5A is blocked; and here, we show that this is because this receptor mutant is unable to interact with AIP4 upon agonist stimulation and is not ubi-

quitinated. We establish that AIP4 is targeted to CXCR4 at the plasma membrane via a novel interaction that involves phosphorylated C-tail serine residues and the WW domains of AIP4. As GPCRs contain a number of serine and threonine residues within their C-tail and are commonly regulated by phosphorylation, WW domain mediated binding may represent a general mode of interaction with GPCRs, suggesting that AIP4 and/or other Nedd4-family members may have a broad role in regulating GPCRs.

In addition to the plasma membrane, we observed increased FRET intensity between CXCR4-YFP and AIP4-CFP in an intracellular location, which is not surprising because we have shown previously that CXCR4 and AIP4 colocalize on endosomes (Marchese *et al.*, 2003). This interaction may also be mediated by phosphorylation of serine residues 324 and 325 as overall cellular FRET intensity between receptor and AIP4 was decreased in the S324/5A expressing cells (Figure 5). However, this intracellular interaction is likely not mediated via simultaneous phosphorylation of these residues because we did not detect intracellular CXCR4 that was simultaneously phosphorylated on S324 and S325 (Figures 8 and 9), suggesting that CXCR4 is dephosphorylated before or after it has internalized onto endosomes. It is important to note that the phospho-serine CXCR4 antibody 5E11 only recognizes simultaneous phosphorylation of residues S324 and S325 and not individually phosphorylated S324 or S325, as determined by confocal immunofluorescence microscopy (Supplemental Figure S2). It may be possible that phosphorylation of serine residue 324 or 325 individually is sufficient to promote AIP4 binding as binding of GST-WW-I-IV to the individual CXCR4 phosphomimetic mutants (S324D and S325D) was also enhanced, compared with the wild-type receptor (Figure 10B). Therefore, AIP4 may interact with endosomal CXCR4 through phosphorylation of either serine residue 324 or 325, but this remains to be explored.

The novel recognition sequence within CXCR4 was surprising given that WW domains are thought to bind to proline-based recognition sequences and that the C-tail of CXCR4 does not contain proline residues. All four WW domains of AIP4 have been shown to bind to proline-rich PY motifs (Winberg *et al.*, 2000; Otte *et al.*, 2003; Ingham *et al.*, 2005b). We report here that WW domains I and II may also recognize nonproline-based sequences. We do not believe this is because WW domains I and II adopt a unique structure that would allow them to bind to nonproline-based domains, because structural studies reveal that at least for WW domain I of Itch (the AIP4 mouse orthologue) the structure is similar to other WW domains (Otte *et al.*, 2003). The ability of a WW domain to recognize multiple motifs is not unprecedented because several WW domains have been shown to interact with multiple recognition sequences (Ingham *et al.*, 2005a). Although it is possible that other regions of AIP4 contribute to the interaction, their role in binding is likely to be minimal given that GST-AIP4ΔWW showed almost no binding to CXCR4.

Our data also reveal several common elements between WW domain recognition of proline-based motifs and of the novel CXCR4 recognition sequence we have defined in this study. Typically, the second conserved tryptophan residue in WW domains is important for proline-residue-based recognition (Zarrinpar and Lim, 2000). Our data indicate that this residue in both WW domains I and II is also important for CXCR4 recognition, although precisely how it participates in the interaction remains to be determined. In addition, we have identified two conserved amino acid residues located at the analogous position in WW domains I (Gln297)

and II (Asn329) that are important for mediating the interaction with CXCR4. Interestingly, these residues are located at the analogous position to an arginine residue located in the WW domain of Pin1 that has been shown to be directly involved in binding to phospho-Ser-Pro motifs (Verdecia *et al.*, 2000). The positively charged group of Arg17 in the Pin1 WW domain makes contact with the negative charge of the phosphorylated Ser residue of the p(Ser)-Pro motif (Verdecia *et al.*, 2000). Although Gln297 and Asn329 carry neutral side chains, it is possible that phosphorylated serine residues 324 and 325 may interact with WW domains I and II through hydrogen bonding with the amide side chains of Gln297 and Asn329, but this will require high resolution structural analysis to confirm. It is interesting to note that WW domains III and IV do not have a glutamine or an asparagine residue at the analogous position, which may explain why these WW domains do not bind to CXCR4, although they have been shown to bind to PY motifs (Winberg *et al.*, 2000; Hu *et al.*, 2004). Therefore it is likely that this novel noncanonical WW domain mediated interaction that we report here may apply to only a subset of WW domains. Interestingly, several WW domains from other Nedd4-like E3s also contain an asparagine or an arginine residue at this critical position (see Figure 4B for an alignment), raising the possibility that these WW domains may bind to serine and/or threonine residues found in a similar context to those present in CXCR4. Regardless, we clearly establish the importance of several AIP4 WW domain residues in mediating the interaction with CXCR4.

In summary, we show here that AIP4 can interact directly with the C-tail of CXCR4 via a novel WW domain recognition sequence that does not contain proline residues. Our data reveal a model whereby agonist activation and phosphorylation of CXCR4 leads to the recruitment of AIP4 to the receptor such that ubiquitination of nearby lysine residues can occur thus enabling the receptor to be targeted for lysosomal degradation. To our knowledge, this study establishes for the first time a mechanism whereby a HECT domain E3 ligase can be directly targeted to an activated GPCR. Whether this will emerge as a general mechanism by which E3 ligases are targeted to GPCRs remains to be determined.

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