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Ubiquitination of Chemokine Receptors

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Abstract

Ubiquitin modification of proteins has traditionally been linked to proteasomal degradation, but it is now well established that it also serves nonproteasomal functions, such as DNA repair, signal transduction and endocytic trafficking among others. It is now emerging that G-protein–coupled receptor (GPCR) downregulation is mediated by receptor ubiquitination. For example, agonist-dependent ubiquitination of the chemokine receptor CXCR4 by the E3 ubiquitin ligase AIP4 (atrophin interacting protein 4) targets CXCR4 for degradation in lysosomes. The ubiquitin moiety on CXCR4 serves as a signal on endosomes for entry into the degradative pathway and long-term attenuation of signaling or downregulation. Several GPCRs have been shown to be ubiquitinated, and ubiquitin-dependent trafficking may represent a general mechanism by which GPCRs are targeted to lysosomes, although some GPCRs that are targeted to lysosomes may not be directly regulated by ubiquitination. Here we describe a simple biochemical assay that we have used to study the ubiquitination of CXCR4 that can be easily applied to study the ubiquitination of any GPCR.

1. Introduction

Chemokine receptors belong to the large superfamily of G-protein–coupled receptors (GPCRs) that are coupled to heterotrimeric G proteins, especially the $G_{\alpha i}$ subfamily, through which a wide variety of intracellular signaling pathways are activated (Busillo and Benovic, 2007). In order to ensure that signals are of the appropriate magnitude and duration signaling is rapidly terminated by a complex series of events giving rise to the phenomenon known as desensitization. Desensitization is a process whereby signaling is attenuated even in the continuous presence of stimulus. Multiple mechanisms contribute to GPCR desensitization, including, in part, the removal of the receptor from the cell surface through a process involving internalization, which sequesters the receptor from its stimulus (Moore *et al*., 2006; Pierce *et al*., 2002). The mechanisms involving GPCR internalization are not completely understood but generally involve receptor phosphorylation by G protein–coupled receptor kinases (GRKs) resulting in arrestin binding and recruitment for internalization through clathrincoated pits (Drake *et al*., 2006; Moore *et al*., 2006). As is true for many GPCRs, chemokine receptors readily undergo ligand-dependent internalization into a vesicular compartment known as an early endosome. Once on early endosomes, GPCRs are subject to an endocytic sorting event that targets them into either a recycling pathway and/or

a degradative pathway (Hanyaloglu and von Zastrow, 2008; Marchese *et al*., 2008). Receptors that enter the recycling pathway are returned to the cell surface, giving rise to receptor resensitization where they are able to respond to further stimulation. Receptors that enter the degradative pathway are targeted to lysosomes for proteolysis, giving rise to long-term attenuation of signaling or downregulation. The mechanisms mediating endosomal sorting remain poorly understood, although for some receptors it appears that sorting into the degradative pathway is mediated by receptor ubiquitination (Hanyaloglu and von Zastrow, 2008; Marchese *et al*., 2008).

We have shown that the CXCR4 chemokine receptor undergoes ligand-dependent post-translational modification by ubiquitin (Marchese and Benovic, 2001). Ubiquitin is a 76–amino-acid protein and is attached to proteins through an ATP-dependent enzymatic process involving three sequential enzymatic steps (Hershko and Ciechanover, 1998; Kerscher *et al*., 2006; Pickart, 2001). The first step is carried out by an E1 enzyme, or activating enzyme, that activates ubiquitin through hydrolysis of ATP leading to the formation of a ubiquitin-adenylate intermediate before ubiquitin is transferred to the active-site cysteine residue of the E1 to form a thiol ester intermediate with the C-terminal glycine residue of ubiquitin. In the second step, ubiquitin is subsequently transferred to the active site cysteine residue of an E2 enzyme, or ubiquitin conjugating enzyme. The third and final step in the process is carried out by the action of an E3 enzyme or ubiquitin ligase. E3 ubiquitin ligases can be broadly classified as falling within two subfamilies, HECT domain and RING finger; they differ in the manner in which ubiquitin is transferred to acceptor lysine residues on the target protein (Hershko and Ciechanover, 1998; Kerscher *et al*., 2006; Pickart, 2001). HECT domain E3s have an active site cysteine residue that forms a direct thiol ester intermediate with ubiquitin before transfer of the ubiquitin moiety to the ε amine group of lysine residues on the target protein. In contrast, RING finger E3s do not form a direct thiol ester intermediate with ubiquitin, but rather, they act as a bridge by bringing the E2 into close proximity to the bound substrate such that transfer of ubiquitin directly from the E2 to the acceptor site lysine residue on the substrate protein can occur. Despite these differences both types of E3s play a significant role in substrate recognition thus providing the substrate specificity associated with ubiquitination reactions. Ubiquitin forms a covalent isopeptide bond with the substrate protein via the carboxyl side group of the terminal glycine residue on ubiquitin and the ε amine group of a lysine residue on the substrate. Ubiquitin attachment is reversible and is subject to removal by deubiquitinating enzymes (Nijman *et al*., 2005). Therefore, the ubiquitination status of a protein at any given time will be dependent upon the relative ratio of ubiquitination/deubiquitination reactions occurring in cells.

We have shown that the chemokine receptor CXCR4 is targeted to lysosomes via a ubiquitin-dependent pathway (Marchese and Benovic, 2001). Agonist-promoted ubiquitination of CXCR4 on carboxyl-terminal tail lysine residues by the E3 ubiquitin ligase AIP4 targets the receptor for degradation in lysosomes (Marchese and Benovic, 2001; Marchese *et al*., 2003b). The ubiquitin moiety on CXCR4 serves as an endosomal signal by likely mediating interactions with core components of the endocytic sorting machinery that contain ubiquitin-binding domains (Marchese *et al*., 2003b). Although CXCR4 has been shown to be modified with ubiquitin and targeted to lysosomes via a ubiquitin-dependent

pathway, this may not occur for all chemokine receptors (Meiser *et al*., 2008). However, whether other chemokine receptors are regulated by ubiquitin in a similar manner to CXCR4 remains to be determined.

One of the easiest methods used to determine whether a protein is modified by ubiquitin is by SDS-PAGE and immunoblotting to detect the protein of interest. Ubiquitin is a 76–amino-acid protein and when conjugated to proteins will add ∼8 kDa to the size of the protein. Therefore, if a protein is ubiquitinated, distinct bands that migrate slower than the unmodified protein by a factor of 8 kDa will indicate the incorporation of one ubiquitin molecule (mono-ubiquitin), 16 kDa for two (di-ubiquitin) and so on. The presence of a smear would suggest that the protein is polyubiquitinated. The unfortunate caveat with this method with respect to GPCRs is the lack of adequate GPCR antibodies to detect the low density of endogenous receptors in many cell types and tissues. Also, the presence of slower migrating bands/smears would still make it difficult to distinguish ubiquitin modification from other types of similar post-translational modifications. Another more commonly used approach relies on the enrichment of the receptor from cells usually by immunoprecipitation followed by SDS-PAGE and immunoblotting with antiubiquitin antibodies to detect incorporated ubiquitin. This approach has been used successfully to detect ubiquitination of CXCR4 (Marchese and Benovic, 2001, 2004; Marchese *et al*., 2003b) as well as other GPCRs (Shenoy *et al*., 2008), although there are caveats associated with this method. One caveat that would apply to CXCR4 (and most GPCRs and/or other proteins) is that because of the low percentage of the total cellular complement of CXCR4 that is ubiquitinated at any given time, we have found it difficult to detect incorporation of endogenous ubiquitin into CXCR4 by using antiubiquitin antibodies. To obviate this difficulty we have resorted to expressing a tagged version of ubiquitin in order to facilitate the ability to detect ubiquitination of CXCR4 (Marchese and Benovic, 2001, 2004; Marchese *et al*., 2003b). Here we describe a method that we have developed to detect ubiquitination of CXCR4 that can also be readily applied to other chemokine receptors or other members of the GPCR family.

2. Cell Culture and Transfections

We use HEK293 (Microbix, Toronto, ON, Canada) and HeLa (ATCC) cells as our model cells to study CXCR4 ubiquitination and trafficking; both cell types express endogenous levels of CXCR4, although we typically use HEK293 cells for heterologous expression studies. We have shown that endogenous CXCR4 is rapidly targeted to lysosomes for proteolysis in CEM cells, a T-cell line; therefore, it appears that endocytic trafficking pathways are conserved among various cell types (Marchese and Benovic, 2001). For ubiquitination assays, we use HEK293 cells and transiently transfect cells with HA-tagged CXCR4 and FLAG-tagged ubiquitin. As discussed above, in our hands it has been difficult to observe ubiquitination of CXCR4 by immunoblotting for endogenous ubiquitin, although others have been successful (Li *et al*., 2004; Zaitseva *et al*., 2005).

We typically grow HEK293 cells on 10-cm tissue culture–grade Petri dishes. HEK293 cells are seeded from a confluent 10-cm dish at a dilution of 1:3 onto a 10-cm dish containing 10 ml culture medium (DMEM supplemented with 10% fetal bovine serum). We find that with

this dilution, the next-day cells are approximately 60 to 70% confluent, which is ideal for transfection. On the day of transfection, the medium is replaced with 10 ml of fresh culture medium. We typically use FuGENE6 transfection reagent (Roche), following the manufacturer's instructions. We cotransfect a total of 10 μg of DNA per 10-cm dish using 30 μl of FuGENE6. For ubiquitination assays, we typically use 7 μg HA-tagged CXCR4 plus 3 μg FLAG-tagged ubiquitin. We have successfully used FLAG-tagged ubiquitin to detect ubiquitinated CXCR4 (Bhandari *et al*., 2007; Marchese and Benovic, 2001; Marchese *et al*., 2003b). As controls, parallel plates are transfected with DNA encoding either receptor or ubiquitin alone. The following day, cells from each transfection should be ∼100% confluent. Transfected cells are seeded onto two 6-cm dishes and allowed to grow for an additional day (∼18 to 24 h). The next day (i.e., day of the experiment), the cells should be approximately ∼90% confluent (∼500,000 cells). Two plates from each transfection condition are plated to allow for treatment with vehicle and agonist. By following this transfection procedure it will permit multiple treatment conditions using cells derived from the same transfection, which will reduce variability owing to differences in expression levels among transfections from plate to plate. The agonist for CXCR4 is stromal-cell–derived factor 1α (SDF-1α), also known as CXCL12, purchased from PeproTech (Rocky Hill, NJ). Single-use aliquots of SDF-1α (10 μ*M*) resuspended in PBS containing 0.1% BSA are stored frozen at –20 °C for up to 3 months.

3. Agonist Treatment and Ubiquitination Assay

On the day of the experiment, cells are washed 1 × with 2 ml warm DMEM and incubated with fresh 1.5 ml DMEM supplemented with 20 m*M* HEPES for ∼3 h. The media is replaced with the same media containing either vehicle (0.1% BSA in PBS) or SDF-1α (100 n*M*; final concentration) in a total volume of 1.5 ml. The cells are treated at 37 °C for 30 min. We typically use SDF-1α at a maximal final concentration of 100 n*M* to ensure full receptor occupancy, enabling maximal receptor ubiquitination to facilitate detection of ubiquitinated CXCR4. We typically treat cells for 30 min because we have determined that under these conditions maximal levels of ubiquitinated CXCR4 are detected (Marchese *et al*., 2003b). Optimal concentrations of ligand and length of treatments will have to be empirically determined for different cell types and/or specific chemokine receptors being examined.

After treatment, plates are immediately placed on ice, media is aspirated and cells are scraped in 800 μl ice-cold lysis buffer (50 m*M* Tris-HCl, pH 8, 150 m*M* NaCl, 5 m*M* EDTA, 0.5% sodium deoxycholate [w/v], 1% non-idet P-40 [NP40, v/v], 0.1% sodium dodecyl sulfate [SDS, w/v], 20 m*M* NEM and protease inhibitors [10 μg/ml each of pepstatin A, leupeptin, aprotinin]). It is important to include NEM to the buffer in order to block free sulfhydryl groups on catalytic-site cysteine residues of deubiquitinating enzymes (DUBs). Active DUBs in cellular lysates may remove ubiquitin attached to proteins, thus making it difficult to detect ubiquitinated proteins. In addition, please note that the lysis buffer we use to immunoprecipitate HA-tagged CXCR4 is somewhat stringent to reduce the likelihood of co-immunoprecipitating proteins that may themselves be ubiquitinated, which could confound data interpretation. We have found that these conditions work best for immunoprecipitating HA-CXCR4 from lysates prepared from HEK293 cells. If different

epitope tags and/or different receptors are used, optimization studies will have to be performed.

Transfer the lysates to fresh microcentrifuge tubes and place at 4 °C while gently rocking for approximately 30 min to ensure complete lysis, followed by sonication on ice for 10 s at setting 10% (Branson Digital Sonifier 450). Samples are clarified by centrifugation at 21,000×g for 20 min at 4 °C. Save an aliquot of the cleared lysate in an equal volume of 2× sample buffer (0.0375 *M* Tris-HCl, pH 6.5, 8% SDS, 10% glycerol, 5% *β*-mercaptoethanol, 0.003% bromophenol blue) for Western blotting to assess the expression of the various constructs. To immunoprecipitate the receptor, incubate 600 *μ*l of the cleared lysate for 1 h with a polyclonal antibody against the HA epitope (HA.11, 1:300 dilution; Covance, Emeryville, CA). Add 20 *μ*l of a 1:1 ratio of protein A equilibrated in lysis buffer and incubate for an additional 1 h at 4 °C while rocking. Briefly wash samples twice with 750 *μ*l lysis buffer. Elute bound proteins with 20 *μ*l 2× sample buffer at room temperature (RT) for 30 min. Because most GPCRs will aggregate when boiled and will not properly separate even under denaturing conditions, it is important not to boil samples that contain GPCRs. We typically use a Hamilton syringe equipped with a 24-gauge needle to load samples on 7% SDS-PAGE, followed by electrophoretic transfer onto nitrocellulose membranes according to the manufacturer's recommendations (Bio-Rad, Hercules, CA). We typically separate proteins using 7% polyacrylamide gels to ensure robust separation and transfer of high-molecular-weight ubiquitinated proteins.

The membrane is blocked for 30 min in 10 ml Tris-buffered saline (TBS) (50 m*M* Tris-HCl, pH 7.4, 150 m*M* NaCl) with 0.05% Tween 20 (v/v) (TBST) containing 5% non-fat dried milk (w/v) at RT while rocking. To detect incorporation of tagged ubiquitin into the receptor the membrane is probed with anti-FLAG M2 monoclonal antibody (5 *μ*g/ml; Sigma) for at least 1 h at RT or overnight at 4 °C while rocking. Wash the membrane at least 3× for 5 min at RT. Incubate the nitrocellulose membrane with 10 ml TBST-5% milk containing goat antimouse IgG conjugated to horseradish peroxidase (HRP) at a dilution of 1:10, 000. Wash the nitrocellulose membrane 5× for 10 min each in TBST. Overlay the nitrocellulose with 1 to 2 ml of Supersignal Chemiluminescence reagent (Pierce, Rockford, IL) for ∼5 min, allow the blot to dry, wrap in plastic wrap, and visualize on x-ray film. Nitrocellulose membranes can be treated with stripping buffer (62.5 m*M* Tris-HCl, pH 6.7, 100 m*M* *β*-mercaptoethanol, 2% SDS) to remove bound antibody and reprobed with a monoclonal anti-HA antibody (HA.11, Covance, Emeryville, CA) to detect receptor levels and to assess loading.

4. E3 Ubiquitin Ligase AIP4 Mediates Ubiquitination of CXCR4

As discussed above, the E3 ubiquitin ligase is one of the most important enzymes involved in the conjugation of ubiquitin to an acceptor lysine residue on the target protein as it provides the specificity associated with ubiquitin reactions while it mediates binding to the target protein. To further understand how CXCR4 is regulated by ubiquitin, we determined that AIP4 (atrophin interacting protein 4), a HECT-domain E3 ubiquitin ligase mediates ubiquitination of CXCR4 (Marchese *et al*., 2003b). To identify AIP4 as an E3 ubiquitin ligase for CXCR4, we took a candidate protein approach. There are ∼600 sequences in the human genome that potentially encode E3 ubiquitin ligases (Li *et al*., 2008), presenting a

daunting to task to identify the E3 that mediates ubiquitination of any protein let alone CXCR4. Information about the possible ligase that may regulate CXCR4 came from studies performed in *S. cerevisiae,* the budding yeast, in which Rsp5 was found to mediate ubiquitination and internalization of the α-mating factor receptor, a GPCR (Dunn and Hicke, 2001). The human genome encodes nine orthologous E3s to yeast Rsp5, which are part of the Nedd4-like family of E3 ubiquitin ligases (Ingham *et al*., 2004). Members of this family are characterized by the presence of a calcium-dependent phospholipid-binding domain, three to four tandemly linked WW domains, and a HECT domain (Ingham *et al*., 2004). In general, the WW domains either directly or indirectly interact with their target proteins typically via PY motifs (i.e., PPXY, PPPY) (Ingham *et al*., 2005). As mentioned above, HECT domain E3s have a catalytic cysteine residue that forms a direct thiol ester intermediate with the terminal glycine residue of ubiquitin before transfer to an acceptor lysine residue on the target protein (Huibregtse *et al*., 1995). Changing the catalytic cysteine residue to a serine or alanine residue creates a catalytically inactive mutant that behaves as a dominant-negative when overexpressed in cells by inhibiting binding of the endogenous E3 to its target and thus inhibiting its activity. Initially, we took a dominant-negative approach to determine whether CXCR4 ubiquitination was regulated by E3s belonging to this family. Members of this family that we have examined include AIP4, Nedd4, and Nedd4-2 (Marchese *et al*., 2003b). HEK293 cells grown on 10-cm dishes are cotransfected as described above with HA-tagged CXCR4 (1 μg) and either wildtype or mutant versions of Nedd4, AIP4, or Nedd4-2 (1 μg) plus FLAG-ubiquitin (1 μg). Cells are treated with vehicle or SDF-1α and ubiquitination of CXCR4 is assessed as described above. Under these conditions, we have observed that cotransfection with AIP4-C830A, a catalytically inactive mutant of AIP4, attenuates agonist-promoted ubiquitination of CXCR4 and that either wildtype or catalytically inactive forms of Nedd4 and Nedd4-2 had no noticeable effect on CXCR4 ubiquitination (Marchese *et al*., 2003b). The amount of DNA to transfect for each construct will have to be empirically determined and titrated accordingly to avoid off-target effects.

Once a candidate E3 is identified by taking the dominant-negative approach, the next step will be to further confirm a specific role for the E3 in the ubiquitination of the receptor of interest by taking a genetic approach (Marchese *et al*., 2003b). We have employed siRNA to reduce endogenous levels of AIP4 in HEK293 and HeLa cells (Marchese *et al*., 2003b). We have used a custom-designed siRNA sequence targeting AIP4 (GenBank Accession No. AF095745): GGU GAC AAA GAG CCA ACA GAG, and corresponds to nucleotides 190 to 211 relative to the start codon. The AIP4 siRNA was synthesized by Dharmacon Research (Lafayette, CO) and is supplied as 23-nucleotide duplexes with 2-nucleotide 3′ (2-deoxy) thymidine overhangs. Control siRNA can be against an irrelevant protein such as luciferase. HEK293 cells are cotransfected with HA-CXCR4 plus FLAG-ubiquitin together either with vehicle, control siRNA, or AIP4-specific siRNA.

Cells are seeded onto 10-cm dishes the day before transfection (about 15 h) such that the confluency at the time of transfection is at least 80%, which appears to have less-toxic effects on the cells when the transfection reagent is applied. Although there are many reagents available for siRNA transfection, we have had great success using Lipofectamine

2000 (Invitrogen, Carlsbad, CA). Add 30 μl of Lipofectamine 2000 to 1.5 ml OPTI-MEM (Invitrogen, Carlsbad, CA) and incubate at RT for 5 min. In another tube, add 1.5 ml OPTI-MEM, DNA encoding receptor (1 μg), tagged ubiquitin (1 μg), and the siRNA equaling 600 pmol. Add the DNA/siRNA/OPTI-MEM mixture dropwise to the tube containing the OPTI-MEM plus Lipofectamine 2000 and incubate at RT for 20 min. Add the mixture drop wise to cells grown on a 10-cm Petri dish containing 3.5 ml culture medium and place at 37 °C for 24 h. The final concentration of the siRNA will be 100 n*M*. After 24 h, passage the cells onto 6-cm dishes and perform the ubiquitination experiments the next day as described above. Also, pass cells into a parallel plate to test for expression of AIP4. The custom AIP4 siRNA significantly (>90%) reduces AIP4 expression compared to vehicle- or control siRNA–treated cells (Marchese *et al*., 2003b). Commercially available siRNA and shRNA targeting AIP4 and other E3 ubiquitin ligases are available from several companies. Recently, Nedd4 has been shown to mediate ubiquitination of the β_2-adrenergic receptor (Shenoy *et al*., 2008), suggesting that members of the Nedd4-like E3 ubiquitin ligases may play a broad role in regulating ubiquitination of GPCRs.

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