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Ubiquitination of GPCRs

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Abstract

In this chapter, we describe a method for detecting the ubiquitination status of G protein-coupled receptors (GPCRs). This involves co-expression of a GPCR with an epitope-tagged ubiquitin construct in a heterologous mammalian expression system. Stimulus-dependent modification of the GPCR by ubiquitin is detected by immunoprecipitation and subsequent immunoblotting to detect incorporation of the epitope-tagged ubiquitin. We describe here a well-established protocol to detect ubiquitination of the chemokine receptor CXCR4, which can be easily applied to detect the ubiquitination status of other GPCRs.

Keywords

Ubiquitin; G protein-coupled receptor; Agonist; Immunoblot; Lysosome; Sorting; Degradation; CXCR4; Immunoprecipitation; De-ubiquitination

1. Introduction

Many agonist-activated G protein-coupled receptors (GPCRs) are subject to a complex series of events that may lead to their removal from the cell-surface and internalization into an endocytic compartment (1, 2). Once on endosomes, internalized receptors are subject to multiple sorting events that ultimately direct them to a recycling pathway and/or a degradative pathway. If a receptor enters the recycling pathway, it will be brought back to the plasma membrane allowing for a process known as resensitization. Alternatively, if a receptor enters the degradative pathway, it will traffic to lysosomes where it will be degraded, giving rise to a process known as downregulation. Very little is known about the molecular mechanisms mediating the sorting events linked to the postinternalization trafficking of GPCRs. However, we have shown that agonist-promoted ubiquitination of the chemokine receptor CXCR4 by the E3 ubiquitin ligase AIP4 is important for targeting the receptor to lysosomes (3, 4). A CXCR4 mutant in which three lysine residues in the C terminus were changed to arginine residues internalized normally, but failed to undergo agonist-promoted degradation (5). Likewise, in cells depleted of AIP4 by RNA interference CXCR4 internalized normally, but was unable to undergo agonist-promoted degradation as compared to control, AIP4-replete cells (4). Both the receptor mutant and AIP4 depletion attenuated CXCR4 ubiquitination, indicating that the ubiquitin moiety is necessary for targeting CXCR4 into a degradative compartment. Other GPCRs may also be targeted to degradative compartments by a similar ubiquitindependent endosomal pathway, although

the ubiquitination machinery may be different (6, 7). Of note, direct ubiquitinmodification of some GPCRs may not necessarily constitute the lysosomal targeting signal (8–10).

Ubiquitin is a highly conserved 76 amino acid polypeptide that is usually covalently attached to proteins through the formation of an isopeptide bond between the C-terminal glycine residue of ubiquitin and the ε-amino group of lysine residues of target proteins (11). Through seven internal lysine residues ubiquitin can then be modified by itself to form polyubiquitin chains. However, the most common polyubiquitin chains observed are formed through linkages at K-48 and K-63 (12). Generally, K-48 linkages target proteins for proteolysis by the proteasome while K-63 linkages or monoubiquitin subserve nonproteasomal functions. Both K-63 and monoubiquitin have been linked to a role in endosomal trafficking of many integral membrane proteins (13, 14). Ubiquitin is involved in the internalization of cell-surface proteins and the sorting of membrane proteins into the multivesicular body (MVB), a morphologically distinct endosomal compartment containing intraluminal vesicles associated with endosomal degradation (15, 16). The ubiquitin moiety on receptors is recognized by proteins of the endosomal sorting complex required for transport (ESCRT) machinery, of which many components harbor ubiquitin binding domains (UBDs), thus providing the framework for a network of ubiquitin/UBD interactions that ultimately leads to the proper sorting and degradation of ubiquitinated cargo in lysosomes (15).

A widely used method to detect the ubiquitination status of a protein is by immunoblotting. The covalent attachment of ubiquitin adds at least 8 kDa to the size of a protein, thereby leading to slower migration when subjected to SDS–PAGE analysis. The ubiquitinated forms can then be detected by immunoblotting using an antibody that recognizes the modified protein, as ubiquitinated forms migrate at ~8 kDa intervals above the unmodified version. However, because the ubiquitinated GPCR may represent only a small fraction of the total membrane pool of the GPCR, antibodies raised against GPCRs are often not sensitive enough to detect the small amount of the ubiquitinated GPCR species. A more sensitive method of detecting the ubiquitination status of a GPCR is to concentrate the receptor, typically by immunoprecipitation, followed by SDS–PAGE and immunoblotting with an antibody against ubiquitin. We describe here a procedure that enables the detection of ubiquitinated chemokine receptor CXCR4. This procedure can readily be adapted and applied to detect the ubiquitination status of other members of the GPCR family.

2. Materials

1. Human embryonic kidney cells (HEK293; American Tissue Type Collection).
2. Dulbecco’s Modified Eagle’s medium (DMEM; Mediatech, Herndon, VA) and fetal bovine serum (FBS; Hyclone, Logan, UT).
3. Stromal-cell derived growth factor-1a (SDF-1a) (PeproTech, Rocky Hill, NJ) dissolved in phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin. Note, SDF-1a is also known as CXCL12.
4. TransIT transfection reagent (Mirrus, Madison, WI) and protein-A agarose beads (Roche, Indianapolis, IN).

5. DNA expression constructs: HA-tagged CXCR4 in pcDNA3 (Invitrogen, Carlsbad, CA), FLAG-tagged ubiquitin in pCMV10 (Sigma, St. Louis, MO) (see Note [1]).
6. PBS (Hyclone, Logan, UT).
7. Lysis/wash/immunoprecipitation buffer: 50 mM Tris–HCl (pH 8.0), 150 mM NaCl, 5 mM EDTA, 0.5% sodium deoxycholate (w/v), 1% Nonidet P-40 (NP40; v/v), 0.1% sodium dodecyl sulfate (SDS; w/v) (see Note [2]).
8. Protease inhibitors are added fresh each time with the following final concentrations: 10 μg/mL aprotinin, 10 μg/mL leupeptin, 0.2 μg/mL benzamidine and 1 μg/mL pepstatin-A. Each inhibitor can be obtained from Roche (Indianapolis, IN).
9. *N*-ethylmaleimide (NEM; Sigma, St. Louis, MO) is added fresh each time to a final concentration of 20 mM (see Note [3]).
10. SDS–PAGE gels: We typically use 7% gels cast before use (see Note [4]).
11. Denaturation solution for immunoblots: 62.5 mM Tris–HCl (pH 6.7), 100 mM β-mercaptoethanol, 2% SDS (see Note [5]).
12. Tris-buffered saline (50 mM Tris–HCl (pH 7.4), 150 mM NaCl) containing 0.05% Tween-20 (TBST). Also, TBST containing 5% (w/v) nonfat dry milk (TBST-5% milk).
13. Antibodies: HRP-labeled or unconjugated anti-FLAG M2 monoclonal antibody (Sigma, St. Louis, MO); anti-mouse IgG conjugated with horseradish peroxidase (if using unconjugated M2 antibody; Vector Laboratories, Burlingame, CA); mouse monoclonal anti-HA and rabbit anti-HA antibodies (Covance, Richmond, CA).
14. 2× sample buffer: 37.5 mM Tris–HCl (pH 6.5), 8% SDS, 10% glycerol, 5% β-mercaptoethanol, 0.003% bromophenol blue.

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[1]Although commercial anti-ubiquitin antibodies are available, we have had no success at detecting endogenous ubiquitin attached to CXCR[4]Thus, in our studies we employ an epitope-tagged version of ubiquitin in order to increase the sensitivity of detection. The presence of the tag does not appear to affect conjugation to substrate proteins (18). We subcloned yeast ubiquitin into p3 × FLAG–CMV-10 expression vector (Sigma; Cat# E4401), which carries an in-frame triple FLAG sequence upstream of the cloning site (5). Myc-tagged (18) and hemagglutinin (19) versions have also been used successfully. Several commercially available anti-ubiquitin antibodies from several sources are currently available that recognize specific types of linkages and/or monoubiquitin (20, 21). The anti-ubiquitin antibody clone P4D1 has been used successfully to detect ubiquitination of the β_2-adrenergic receptor (22).

[2]The recipe for immunoprecipitation buffers varies depending on the receptor being immunoprecipitated. Ideally, high stringency immunoprecipitation and washing conditions are preferred in order to reduce the likelihood of immunoprecipitating nonspecific proteins that may confound the interpretation of ubiquitin immunoblots. An additional and extremely powerful control would be to identify ubiquitin-deficient mutants to assure that the detection of ubiquitinated receptor species is not confounded by the presence of a co-immunoprecipitating protein that may itself be ubiquitinated.

[3]As with other types of posttranslational modifications, ubiquitin attachment to proteins is highly dynamic and a tightly regulated process. Once attached to protein, ubiquitin may be removed by a specialized set of proteases known as deubiquitinating enzymes (DUBs), most of which have active site cysteine residues (23). One function of these enzymes is to recycle ubiquitin. Therefore, the ubiquitinated status of a protein is determined by the rate of ubiquitin conjugation and deconjugation. Because the activity of these enzymes can compromise the ability to detect ubiquitinated proteins, it is critical that they are inhibited when a cell lysate is prepared. Many DUBs contain active sulfhydryl groups that can be blocked by alkylating agents, such as iodoacetamide and NEM. Iodoacetamide can be used at a final concentration of 1 mg/mL. We have used NEM at a final concentration as high as 20 mM. These agents should be added fresh to the lysis buffer just before use.

[4]We typically use 7% gels because it helps resolve the higher molecular weight species of ubiquitinated receptor.

[5]Even though proteins subjected to SDS–PAGE are significantly denatured, we have found that further treatment of blots in this denaturating solution sometimes enhances the ability to detect ubiquitinated receptor. Thus, nitrocellulose membranes can be incubated with denaturation solution for 30 min at 60°C followed by extensive washing with TBST before blocking and immunoblotting for the detection of ubiquitin.

15. A high sensitivity chemiluminescent substrate, such as SuperSignal® West Dura Extended Duration substrate (Pierce, Rockford, IL).

3. Methods

1. HEK293 cells are maintained in DMEM supplemented with 10% FBS without antibiotics.
2. The day before transfection, passage cells onto 10-cm dishes so that they reach 50–60% confluency on the day of transfection (see Note [6]).
3. Transiently transfect cells with 1 μg of DNA encoding HA-CXCR4, 3 μg of FLAG–ubiquitin and 6 μg of empty vector (pCMV or pcDNA, for a total of 10 μg of DNA per 10-cm dish/transfection) using the TransIT-LT1 transfection reagent, according to the manufacturer's instructions (see Note [7]).
4. Passage cells onto 6-cm dishes 24–48 h after FLAG–ubiquitin transfection (typically at 1:4 to 1:5 ratio) (see Note [8]).
5. Wash cells by aspirating medium and replacing it with 4 mL of warm DMEM supplemented with 20 mM HEPES, pH 7.4. Repeat this step for a total of two washes.
6. Incubate cells in the same medium in the presence or absence of agonist (10–100 nM of SDF1-α in a volume of 1.5 mL) for 15–30 min at 37°C (see Note [9]).
7. Rapidly wash cells once with 2 mL of ice-cold PBS. Aspirate PBS completely and place dishes on ice.
8. Add 1 mL of ice-cold lysis buffer. Scrape cells, and transfer to microcentrifuge tubes. Incubate tubes on ice for ~20 min to allow for complete solubilization (see Note [10]).

[6]We typically perform experiments 48–72 h after FLAG-ubiquitin transfection. Therefore, cells should be passaged at an appropriate density such that they are not overgrown the day of the experiment. This variable should be determined empirically.

[7]For the yeast α-mating factor receptor, the ability to detect ubiquitinated receptor is enhanced in a yeast strain that is defective in clathrin-mediated endocytosis (24). This is due in part to the ubiquitination reaction occurring at the plasma membrane, thus increasing the proportion of receptors that are modified by ubiquitin, but also because it prevents the receptor from entering a compartment where it is de-ubiquitinated (25). Similarly, co-expression of 1 μg of the dynamin dominant- negative K44A mutant, which inhibits CXCR4 internalization, facilitates the detection of ubiquitinated CXCR4 (5). This is potentially useful for the enrichment of ubiquitinated forms of other GPCRs when detection is problematic (26).

[8]We have determined that overexpression of epitope-tagged ubiquitin is the most important variable in the successful detection of ubiquitinated receptors. High expression levels can be achieved by allowing an extra day for FLAG-ubiquitin expression (72 h posttransfection) and/or by maximizing the delivery of plasmids with high-performance/cell-specific transfection reagents (e.g., Mirrus TransIT-293 for HEK293 cells). This should be empirically determined for each cell-type used.

[9]We initially performed a time-course to determine the optimal length of stimulation that resulted in maximal ubiquitination of CXCR[4]Depending on the treatment/agonist concentration, ubiquitination can be detected as early as 10 min. We found that 30 min of stimulation led to the greatest ubiquitination under our conditions; however, this has to be determined for other GPCRs.

[10]Membrane solubilization is a particularly important variable in the successful completion of ubiquitination experiments. We have determined that a 70–90% confluent 6-cm dish of HEK293 cells is efficiently solubilized with 1 mL of our lysis buffer. Importantly, increasing cell number or decreasing lysate volume (thus effectively increasing lysate protein concentration) might even be detrimental to ubiquitin detection by altering CXCR4 solubilization. Because this varies depending on the GPCR and cell-type used, we highly recommend empirically to determine the most efficient solubilization conditions for the cell-type and number of cells to be utilized. In addition, longer incubations while rocking at 4°C can also be performed to allow for complete membrane solubilization.

9. Sonicate samples at 11% power using a microtip probe (Branson Digital Sonifier; Branson, Danbury, CT) for 10 s on ice (to minimize de-ubiquitination and/or proteolysis).

10. Pellet cellular debris by centrifuging samples at 16.3 × 1000 *g* for 20 min in a refrigerated table-top microcentrifuge (4°C).

11. Carefully transfer 500 mL of supernatant to a fresh microcentrifuge tube. Add 2.5 mL polyclonal anti-HA antibody and incubate for 1 h while rocking at 4°C (see Note [11]).

12. Add 50 mL of protein A-agarose (50% slurry, previously equilibrated with lysis buffer) and continue incubation for an additional 1 h while rocking at 4°C.

13. Collect receptor-protein A-agarose complexes by centrifugation in a microcentrifuge at 16.3 × 1000 *g* for 5 s.

14. Carefully remove supernatant, resuspend beads in 750 μL of lysis buffer and incubate for 5 min at 4°C while rocking.

15. Repeat steps 13 and 14 two more times.

16. Collect complexes as in step 13 and carefully aspirate the last traces of lysis buffer using a vacuum line. Avoid aspirating pelleted beads.

17. Elute proteins from agarose beads by adding 25 μL of 2× SDS-gel sample buffer followed by gently vortex-mixing. Incubate samples for 30 min at room temperature before proceeding to the next step (see Note [12]). Alternatively, samples can be stored at −20°C or −80°C until further processing.

18. Resolve proteins by SDS–PAGE and transfer to nitrocellulose membranes using a standard Western blot protocol (17) (see Note [4]).

19. Block the nitrocellulose membrane for 30 min at room temperature in 15 mL of TBST containing 5% (w/v) nonfat dry milk.

20. Incubate membrane with 10 mL of TBST-5% milk containing a 1:2,000 dilution of monoclonal anti-FLAG antibody (M2–HRP) for 1 h at room temperature (see Note [13]).

21. Wash the nitrocellulose membrane five times, each for 10 min, in TBST.

22. Overlay the nitrocellulose with 1–2 mL of chemiluminescence reagent for ~5 min, allow the blot to drip dry, wrap in plastic wrap and visualize on X-ray film.

[11]We have been able to successfully immunoprecipitate and detect ubiquitinated CXCR4 from 100 to 250 μg of lysate prepared as described above. For more accurate comparisons between samples, protein amounts in lysates can be quantified using BCA assay (Pierce, Rockford, IL). In addition, incubation times should be kept to a minimum following lysate generation due to the high activity of DUBs present in the lysate.

[12]It is critical that samples are not boiled to elute CXCR4 off of the beads. Boiling causes receptors to aggregate and impede their migration on SDS–PAGE.

[13]The unconjugated mouse anti-FLAG (M2) can also be used for additional signal amplification and detection of ubiquitin with equivalent results, followed by incubation with a goat anti-mouse IgG conjugated to horseradish peroxidase (1:10,000).

23. At this point blots can be treated with the denaturation solution to remove bound antibody and reprobed with the monoclonal HA antibody to detect receptor levels.

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References

1. Marchese A, Paing MM, Temple BR, Trejo J. G protein-coupled receptor sorting to endosomes and lysosomes. Annu Rev Pharmacol Toxicol. 2008; 48:601–629. [PubMed: 17995450]
2. Hanyaloglu AC, von Zastrow M. Regulation of GPCRs by endocytic membrane trafficking and its potential implications. Annu Rev Pharmacol Toxicol. 2008; 48:537–568. [PubMed: 18184106]
3. Bhandari D, Robia SL, Marchese A. The E3 ubiquitin ligase atrophin interacting protein 4 binds directly to the chemokine receptor CXCR4 via a novel WW domain-mediated interaction. Mol Biol Cell. 2009; 20:1324–1339. [PubMed: 19116316]
4. Marchese A, Raiborg C, Santini F, Keen JH, Stenmark H, Benovic JL. The E3 ubiquitin ligase AIP4 mediates ubiquitination and sorting of the G protein-coupled receptor CXCR4. Dev Cell. 2003; 5:709–722. [PubMed: 14602072]
5. Marchese A, Benovic JL. Agonist-promoted ubiquitination of the G protein-coupled receptor CXCR4 mediates lysosomal sorting. J Biol Chem. 2001; 276:45509–45512. [PubMed: 11641392]
6. Jacob C, Cottrell GS, Gehringer D, Schmidlin F, Grady EF, Bunnett NW. c-Cbl mediates ubiquitination, degradation, and down-regulation of human protease-activated receptor 2. J Biol Chem. 2005; 280:16076–16087. [PubMed: 15708858]
7. Shenoy SK, Xiao K, Venkataramanan V, Snyder PM, Freedman NJ, Weissman AM. Nedd4 mediates agonist-dependent ubiquitination, lysosomal targeting, and degradation of the β_2-adrenergic receptor. J Biol Chem. 2008; 283:22166–22176. [PubMed: 18544533]
8. Tanowitz M, Von Zastrow M. Ubiquitination-independent trafficking of G protein-coupled receptors to lysosomes. J Biol Chem. 2002; 277:50219–50222. [PubMed: 12401797]
9. Baugher PJ, Richmond A. The carboxyl-terminal PDZ ligand motif of chemokine receptor CXCR2 modulates postendocytic sorting and cellular chemotaxis. J Biol Chem. 2008; 283:30868–30878. [PubMed: 18755694]
10. Meiser A, Mueller A, Wise EL, McDonagh EM, Petit SJ, Saran N, Clark PC, Williams TJ, Pease JE. The chemokine receptor CXCR3 is degraded following internalization and is replenished at the cell surface by de novo synthesis of receptor. J Immunol. 2008; 180:6713–6724. [PubMed: 18453591]
11. Weissman AM. Themes and variations on ubiquitylation. Nat Rev Mol Cell Biol. 2001; 2:169–178. [PubMed: 11265246]
12. Ikeda F, Dikic I. Atypical ubiquitin chains: new molecular signals. EMBO Rep. 2008; 9:536–542. [PubMed: 18516089]
13. Bonifacino JS, Weissman AM. Ubiquitin and the control of protein fate in the secretory and endocytic pathways. Annu Rev Cell Dev Biol. 1998; 14:19–57. [PubMed: 9891777]
14. Hicke L. Protein regulation by monoubiquitin. Nat Rev Mol Cell Biol. 2001; 2:195–201. [PubMed: 11265249]
15. Raiborg C, Stenmark H. The ESCRT machinery in endosomal sorting of ubiquitylated membrane proteins. Nature. 2009; 458:445–452. [PubMed: 19325624]
16. Clague MJ, Urbe S. Multivesicular bodies. Curr Biol. 2008; 18:R402–R404. [PubMed: 18492464]
17. Mundell SJ, Orsini MJ, Benovic JL. Characterization of arrestin expression and function. Methods Enzymol. 2002; 343:600–601. [PubMed: 11665594]
18. Ellison MJ, Hochstrasser M. Epitope-tagged ubiquitin. A new probe for analyzing ubiquitin function. J Biol Chem. 1991; 266:21150–21157. [PubMed: 1718971]

19. Courbard JR, Fiore F, Adelaide J, Borg JP, Birnbaum D, Ollendorff V. Interaction between two ubiquitin-protein isopeptide ligases of different classes, CBLC and AIP4/ITCH. J Biol Chem. 2002; 277:45267–45275. [PubMed: 12226085]
20. Wang H, Matsuzawa A, Brown SA, Zhou J, Guy CS, Tseng PH, Forbes K, Nicholson TP, Sheppard PW, Hacker H, Karin M, Vignali DA. Analysis of nondegradative protein ubiquitylation with a monoclonal antibody specific for lysine-63-linked polyubiquitin. Proc Natl Acad Sci USA. 2008; 105:20197–20202. [PubMed: 19091944]
21. Fujimuro M, Sawada H, Yokosawa H. Production and characterization of monoclonal antibodies specific to multi-ubiquitin chains of polyubiquitinated proteins. FEBS Lett. 1994; 349:173–180. [PubMed: 7519568]
22. Shenoy SK, McDonald PH, Kohout TA, Lefkowitz RJ. Regulation of receptor fate by ubiquitination of activated β_2-adrenergic receptor and β-arrestin. Science. 2001; 294:1307–1313. [PubMed: 11588219]
23. Wilkinson KD. Ubiquitination and deubiquitination: targeting of proteins for degradation by the proteasome. Semin Cell Dev Biol. 2000; 11:141–148. [PubMed: 10906270]
24. Hicke L, Zanolari B, Riezman H. Cytoplasmic tail phosphorylation of the alpha-factor receptor is required for its ubiquitination and internalization. J Cell Biol. 1998; 141:349–358. [PubMed: 9548714]
25. Amerik AY, Nowak J, Swaminathan S, Hochstrasser M. The Doa4 deubiquitinating enzyme is functionally linked to the vacuolar protein-sorting and endocytic pathways. Mol Biol Cell. 2000; 11:3365–3380. [PubMed: 11029042]
26. Wolfe BL, Marchese A, Trejo J. Ubiquitination differentially regulates clathrin-dependent internalization of protease-activated receptor-1. J Cell Biol. 2007; 177:905–916. [PubMed: 17535965]