Regulation of V2 Vasopressin Receptor Degradation by Agonist-promoted Ubiquitination*

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The seven-transmembrane-spanning vasopressin V2 receptor (V2R) is a Gs-coupled receptor that is rapidly phosphorylated and internalized following stimulation with the agonist, arginine-vasopressin. Herein, we show that the V2R is ubiquitinated following agonist stimulation. V2R-ubiquitination is not observed in a β-arrestin1,2 deleted mouse fibroblast cell line and is restored following introduction of β-arrestin2, thus indicating that β-arrestin2 is required for the ubiquitination of V2R. A mutant V2R (K268R) that is not ubiquitinated still activates Gs and internalizes with similar kinetics as the wild type receptor. Unstimulated wild type and K268R mutant receptors degrade at similar rates and have comparable half-lives of 217 ± 17 and 245 ± 29 min as determined by pulse-chase experiments. However, following agonist stimulation, the rate of receptor degradation for the wild type is enhanced (half-life of 69 ± 19 min), whereas that of the mutant is only minimally affected (half-life of 188 ± 11 min). These data suggest that V2R levels are regulated through at least two processes. In the absence of agonist stimulation, a slow degradative pathway operates that is independent of receptor ubiquitination. However, receptor stimulation leads to rapid β-arrestin2-dependent ubiquitination of the receptor and increased degradation.

The vasopressin V2 receptor (V2R) is a seven-transmembrane-spanning receptor that is present primarily in the renal distal tubules and collecting ducts. Interaction with its ligand, arginine vasopressin (AVP), leads to activation of Gs and consequent activation of adenyl cyclase that increases cAMP levels in cells. A cascade of events following the elevated levels of cAMP in kidney cells leads to an increase in the water permeability of the collecting ducts and facilitates water reabsorption (1). Exposure of V2R to its ligand also triggers receptor phosphorylation, internalization, and desensitization resulting in a reduction of the cellular response to the agonist (2, 3). The carboxyl terminus of the human V2R, similar to other G protein-coupled receptors, contains multiple phosphorylation sites and is a major regulatory domain controlling receptor interaction with β-arrestins and their trafficking in the cell (2, 4). Previous studies have shown that V2R interacts tightly with β-arrestin1 or -2 following phosphorylation (4). The receptor β-arrestin complex is then endocytosed via clathrin-coated pits and accumulated in large endocytic vesicles in the proximity of the nucleus. V2R undergoes rapid agonist-promoted endocytosis but appears to be poorly recycled. The intracellularly retained receptor is either slowly recycled to the surface or is degraded.

The 26 S proteasome and lysosomes are two major protein degradative pathways in cells. Lysosomes are vesicular organelles with decreased internal pH that can facilitate the breaking of peptide bonds. Proteasomes are multisubunit complexes consisting of a regulatory and a core particle that can recognize and degrade polyubiquitinated proteins (5). Proteins are targeted to the proteasome via covalent attachment of polyubiquitin chains consisting of at least four ubiquitin peptides attached at specific lysine residues (6). The regulatory particle of proteasome is responsible for the recognition and recycling of polyubiquitin chains back into the cytoplasm in addition to unfolding and threading the unfolded protein through the core particle of the proteasome. The core particle of proteasome cleaves proteins into small peptides ranging in size from 3 to 23 amino acids. Although the initial function assigned to ubiquitination was the targeting of proteins to proteasomes, recent studies indicate that ubiquitination of several membrane proteins serves as a targeting or sorting signal through the endocytic route leading either to the vacuole in yeast or to lysosomes in mammalian cells.

Agonist-promoted ubiquitination of several seven-transmembrane-spanning receptors has been shown to play a key role in either their endocytic trafficking or receptor degradation. For example, monoubiquitination of the yeast pheromone receptor promotes ligand-induced endocytosis and is essential for the targeting of the receptor to the vacuole for degradation (7). Several mammalian seven-transmembrane-spanning receptors such as CXCR4 and β2-adrenergic receptors are also ubiquitinated following agonist stimulation. Mutant versions of these receptors, which cannot undergo ubiquitin modification, internalize but are not degraded (8, 9). In the case of the β2-adrenergic receptor, ubiquitination of β-arrestin is necessary for receptor internalization but apparently not for receptor degradation.

Here we sought to investigate the role of V2 vasopressin receptor ubiquitination in receptor trafficking and degradation. Our findings indicate that V2R is ubiquitinated in an agonist and β-arrestin-dependent manner at a single lysine residue (lysine 268) in the third intracellular loop and that this ubiquitination forces the V2R to a rapid degradative pathway.

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‡ The abbreviations used are: V2R, vasopressin V2 receptor; AVP, arginine vasopressin; CXCR4, chemokine receptor; HA, hemagglutinin; GFP, green fluorescent protein; Vps, vascular protein sorting; TSG101, tumor suppressor gene 101; Hrs, hepatocyte growth factor-regulated tyrosine kinase substrate; Eps15, EGFR pathway substrate clone 15; HEK, human embryonic kidney; E2, ubiquitin carrier protein; E3, ubiquitin-protein isopeptide ligase; MEF, mouse embryonic fibroblast.
Agnost-promoted Ubiquitination of V2 Receptor

Materials—LipofectAMINE was from Invitrogen. HA affinity agarose beads were purchased from Covance or Sigma. Arginine-vasopressin and MG132 were from Sigma. Ubiquitin antibody UbP4D1 and anti-HA rabbit polyclonal antibody were from Santa Cruz Biotechnologies. Easy tag™ express protein labeling mix, 35S, and [3H]Arg-vasopressin were from PerkinElmer Life Sciences.

Cell Culture and Transfection—COS-7 and HEK-293 cells were maintained in Dulbecco’s modified Eagle’s medium (Sigma) and minimal essential medium (Sigma), respectively, and supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. Transient transfection of COS-7 cells was carried out with LipofectAMINE reagent. HEK-293 cells were transiently transfected with FuGENE (Roche).

Receptor Immunoprecipitation and Western Blotting—COS-7 cells were transiently transfected with HA-tagged V2R and FLAG-tagged β-arrestin2. Twenty-four hours after transfection, cells were split into appropriate plates for ubiquitination and binding assays. Forty-eight hours following transfection, cells were serum-starved for 2 h (with or without 10 μM MG132 where appropriate) and then stimulated for the designated amount of time with 1 μM AVP. Cells were lysed using a buffer containing 50 mM HEPES (pH 7.5), 0.5% Nonidet P-40, 250 mM NaCl, 2 mM EDTA, 10% (v/v) glycerol, 1 mM sodium orthovanadate, 1 mM sodium fluoride, 1 mM phenylmethylsulfonyl fluoride, leupeptin (5 μg/ml), aprotonin (5 μg/ml), pepstatin A (1 μg/ml), and 100 μM benzamidine. Cell lysates were incubated with HA beads overnight at 4 °C. The next day, the cell lysate was removed, and the beads were washed four times with the lysis buffer to eliminate nonspecific binding.

RESULTS

AGONIST-PROMOTED UBIQUITINATION OF V2 RECEPTOR

Vasopressin receptor is ubiquitinated upon agonist stimulation. As shown in Fig. 1a, HA-tagged V2R was transiently transfected in COS-7 cells. Cells were serum-starved for 1 h prior to stimulation with 1 μM AVP. V2R was immunoprecipitated and followed by Western blot analysis. The ubiquitinated receptor was visualized using antibodies against ubiquitin (upper panel). Receptor ubiquitination was detected only in the presence of MG132 in AVP-stimulated cells. The same blots were stripped and reprobed using antibodies against the HA tag on the receptor (lower panel). b, time course of vasopressin receptor ubiquitination. Transiently transfected COS-7 cells were stimulated for 5, 15, 30, 60, and 120 min with 1 μM AVP. Immunoprecipitated receptor was subjected to Western blot analysis and probed with an antibody against ubiquitin (upper panel). Ubiquitin blots were stripped and reprobed with anti-HA antibody for receptor levels. Each blot is representative of three independently performed experiments. Receptor expression levels varied between 1.0 and 1.5 pmol/mg of protein as determined by binding assays.

Binding assay—Twenty-four hours after transfection, cells were split into 12-well plates. Forty-eight hours following transfection, cells were washed twice with a wash buffer consisting of Dulbecco’s modified Eagle’s medium, 2% bovine serum albumin, and 1 mM HEPES (pH 7.5). Cells were incubated with radiolabeled solution [3H]Arg-vasopressin (or a mixture of radioligand and 10 μM AVP) for 1 h at room temperature. All wells were washed thrice with the wash buffer to remove all nonspecific binding. Cells were lysed with a solution containing 0.1% SDS and 0.1 M NaOH. The amount of radioactivity retained in cells was measured and receptor concentration was calculated using GraphPad software.

cAMP assay—A [3H]-labeled cAMP assay system (Amersham Biosciences) was used to determine the amount of accumulated cAMP response in transiently transfected cells following stimulation as per previously described protocol (10).

Internalization assay—Twenty-four hours after transfection with HA-tagged V2R and FLAG-tagged β-arrestin2, COS-7 cells were split into appropriate plates. Forty-eight hours following transfection, cells were placed on ice and washed thrice with the wash buffer (Dulbecco’s modified Eagle’s medium, 1% bovine serum albumin, and 1 mM HEPES). Radioligand [3H]vasopressin was added to cells, and cells were placed on a 37 °C hot plate for indicated times to allow for receptor internalization. Cells were washed three times to remove unbound radioligand. Bound cell surface ligand was acid washed, collected, and quantified to determine the amount of receptor that remained on the surface. Following the acid wash, cells were lysed with solution containing 0.1% SDS and 0.1 M NaOH, and the amount of internalized receptor was determined. As a control, binding assays (as described above) were performed simultaneously to determine the total number of receptors on the cell surface prior to internalization assay.

Confocal Microscopy—HEK-293 cells were transfected with HA-tagged V2R and GFP-tagged β-arrestin2 using FuGENE. Twenty-four hours following transfection, cells were split and plated on collagen-coated 35-mm glass-bottom plates and serum-starved for several hours. After addition of an agonist (1 μM AVP), images were obtained at different time points ranging from 0 to 60 min poststimulation on a Zeiss LSM510 laser scanning microscope. Receptor expression was confirmed by radioligand binding.

Pulse-Chase assay—COS-7 cells were transiently transfected with HA-tagged V2R and FLAG-tagged β-arrestin2. Forty-eight hours after transfection, cells were washed twice with phosphate-buffered saline...
and starved in serum-free medium lacking Met and Cys. 100 μCi of Easy tag™ express protein labeling mix, 35S, per milliliter of serum-free buffer (2 ml/10-cm plate), was used to metabolically label the cells for 90 min. Cells were washed with phosphate-buffered saline three times to remove unbound label. The medium was replaced with serum-free Dulbecco’s modified Eagle’s medium, and cells were stimulated with 1 μM AVP for the indicated amount of time. Cells were lysed, and the HA-tagged receptors were immunoprecipitated and run on SDS-polyacrylamide gels as described above. Dried gels were exposed to x-ray film. The bands on the autoradiograph were quantitated using a Bio-Rad Flour-S imager.

RESULTS AND DISCUSSION

To investigate whether human V2R is ubiquitinated following agonist stimulation, COS-7 cells were transiently transfected with HA-tagged V2R and stimulated for 1 h with the agonist, AVP. As shown in Fig. 1a (upper panels), V2R is ubiquitinated following agonist stimulation. The ubiquitinated receptor is detected as a smear on Western blots because of the variable number of conjugated ubiquitin moieties and could only be detected in the presence of the proteasomal inhibitor.

![Diagram of the V2 receptor](https://example.com/v2-receptor-diagram.png)

**Fig. 3. Residue Lys-268 of the V2R is ubiquitinated upon agonist stimulation.** a, schematic representation of the human V2 vasopressin receptor (23). Black arrows point to possible ubiquitination sites on the receptor. b, COS-7 cells were transiently transfected with the wild type (WT), K268R, or K367R V2R. Receptor ubiquitination was visualized on Western blots as described in Fig. 1. Binding assays determined the receptor expressions at 2.3, 1.0, and 0.2 pmol/mg of protein for wild type, K268R, and K367R, respectively. The blots are representatives of three independent experiments. IP, immunoprecipitate; IB, immunoblot.
MG132 that prevents the rapid degradation of the polyubiquitin-conjugated proteins. A time course study determined that after agonist stimulation, V2R ubiquitination was apparent as early as 60 min (Fig. 1b, upper panel) and was prominent at 120 min. For each agonist-stimulated sample, the amount of V2R in the immunoprecipitates was determined by blotting for the HA tag on the receptor (Fig. 1b, lower panel). No changes in the receptor levels were detected after stimulation with AVP.

Detection of V2R ubiquitination requires preincubation of cells with MG132 unlike the β2-adrenergic receptor (8). The known role of MG132 is the inhibition of proteasomal activity. This is suggestive of a degradative role for the proteasomes in the case of V2R apart from the previously implied role in endosomal sorting of cell surface receptor (16). Alternatively, ubiquitination of the V2R may require stabilization of an unknown protein that is rapidly targeted to the proteasomes for degradation.

Previous studies have shown that β-arrestin2 is necessary for β2-adrenergic receptor ubiquitination (8). β2-Adrenergic receptor belongs to a class of receptors (class A) that bind GPF-β-arrestin proteins transiently during endocytosis and have higher affinity for the β-arrestin2 isoform (11). However, V2R classifies as a second group of receptors (class B), which bind β-arrestin more stably and show equal affinity to β-arrestin1 and -2. To determine the involvement of a specific β-arrestin isoform in V2R ubiquitination, we performed experiments in β-arrestin1/2 double knock-out mouse embryonic fibroblast cell lines that were stably transfected with either vector pCDNA3 (MEF-βarr1−/−βarr2−) or FLAG-tagged β-arrestin1 (MEF-βarr1), or β-arrestin2 (MEF-βarr2) (12, 13). All the above three cell lines were transiently transfected with the HA-tagged V2R and assayed for receptor ubiquitination. Receptor ubiquitination was only observed in the MEF-βarr2 cell lines implicating β-arrestin2 as an essential factor in V2R ubiquitination (Fig. 2). β-Arrestin2 is also necessary for ubiquitination of β2-adrenergic receptor. Perhaps, β-arrestin2 serves as an adaptor protein necessary for the recruitment of E2 or E3 enzymes of the ubiquitination machinery that can ubiquitinate the receptor.

Next, we determined the site of ubiquitination in the V2R. Ubiquitin is covalently attached to lysine residues on proteins. Human V2R contains four lysine residues (Fig. 3a). Based on the predicted structure of human V2R, Lys-100 and Lys-116 do not face the cytosol and are thus unlikely to be ubiquitinated. We suspected that the site of ubiquitination of the receptor was on residue Lys-268 that is located in the third intracellular loop of the receptor, which is a predicted region for ubiquitination and proteasomal degradation (17, 18). This is suggestive of a degradative role for the proteasomes in the case of V2R.

Graphed data are representative of four independent experiments. Error bars depict ± S.E. WT, wild type.
receptor degradation in the absence of stimulation. Pulse-chase under "bolic labeling, HA-tagged receptors were immunoprecipitated at different rate of receptor degradation and receptor half-lives. Following metastatic labeling, the receptor levels at time point 0 (immunopre- cipitated immediately following metabolic labeling). Wild type and mu-
tant vasopressin receptor degradation data were fitted with exponential decay curves and analyzed using GraphPad software. No significant differences between the degradation rates of the wild type and the mutant vasopressin receptor were detected (assessed by performing two-way analysis of variance on the graph data). Each graph represents the result of three independent experiments. b, receptor levels were determined up to 6 h following stimulation with AVP. Data were fitted with exponential decay curves. The degradation rate of the wild type vasopressin receptor is considerably increased. Differences in the wild type and the mutant receptor (K268R) degradation are especially profound for the first 2 h following stimulation. Analysis of graph data with two-way analysis of variance confirmed the differences between the two graphs (p < 0.005). Five independent experiments yielded similar results, which are reflected on the graphs. c, GraphPad software was used to analyze the data and fit curves to include all the experimental points. The generated curve for each independent experiment was used to determine the time point at which 50% of receptor remains. The half-lives from individual experiments were then used to generate the bar graphs. Half-lives of receptors were compared using a two-tailed t test. No significant differences between the half-lives of the wild type and the mutant were detected in unstimulated cells. The calculated half-life of the K268R mutant was lowered from 217 ± 17 to 69 ± 19 min following AVP treatment. On the other hand, modest reduction in the half-life of the nonubiquitinated mutant receptor, from 245 ± 29 to 188 ± 11 min, was observed. The half-lives of the wild type and the K268R mutant are significantly different after stimulation with AVP (**, p < 0.005). NS, nonstimulated.

**Fig. 5. Effects of mutation of the V2R ubiquitination site on receptor degradation in the absence of stimulation.** Pulse-chase experiments were performed on transiently transfected COS-7 cells with either wild type (wt) or K268R mutant receptor to determine the rate of receptor degradation and receptor half-lives. Following metabolic labeling, HA-tagged receptors were immunoprecipitated at different time points and analyzed as described under "Pulse-Chase Assay" under "Experimental Procedures." a, receptor levels were determined up to 13 h following metabolic labeling. The density of bands corresponding to each receptor for different time points was normalized against the band for the receptor levels at time point 0 (immunoprecipitated immediately following metabolic labeling). Wild type and mutant vasopressin receptor degradation data were fitted with exponential decay curves and analyzed using GraphPad software. No significant differences between the degradation rates of the wild type and the mutant vasopressin receptor were detected (assessed by performing two-way analysis of variance on the graph data). Each graph represents the result of three independent experiments. b, receptor levels were determined up to 6 h following stimulation with AVP. Data were fitted with exponential decay curves. The degradation rate of the wild type vasopressin receptor is considerably increased. Differences in the wild type and the mutant receptor (K268R) degradation are especially profound for the first 2 h following stimulation. Analysis of graph data with two-way analysis of variance confirmed the differences between the two graphs (p < 0.005). Five independent experiments yielded similar results, which are reflected on the graphs. c, GraphPad software was used to analyze the data and fit curves to include all the experimental points. The generated curve for each independent experiment was used to determine the time point at which 50% of receptor remains. The half-lives from individual experiments were then used to generate the bar graphs. Half-lives of receptors were compared using a two-tailed t test. No significant differences between the half-lives of the wild type and the mutant were detected in unstimulated cells. The calculated half-life of the wild type receptor was lowered from 217 ± 17 to 69 ± 19 min following AVP treatment. On the other hand, modest reduction in the half-life of the nonubiquitinated mutant receptor, from 245 ± 29 to 188 ± 11 min, was observed. The half-lives of the wild type and the K268R mutant are significantly different after stimulation with AVP (**, p < 0.005). NS, nonstimulated.

**β-Arrestin2 interaction with the V2R is crucial for receptor ubiquitination (Fig. 2).** Therefore, impaired interaction of K268R with β-arrestin2 might explain the lack of ubiquitination of the receptor mutant. To investigate this possibility, HEK-293 cells were transiently transfected with GFP-tagged β-arrestin2 and either wild type or K268R mutant receptor. Intact cells were stimulated for 40 min with AVP, and the recruitment of GFP-β-arrestin2 to the endocytic vesicles was observed using confocal microscopy. GFP-β-arrestin2 was uniformly distributed throughout the cytoplasm prior to agonist stimulation (Fig. 4b). Following stimulation, GFP-β-arrestin2 was recruited to the plasma membrane and eventually accumulated within the cell in endocytic vesicles (Fig. 4b). This phenomenon has been described for several G protein-coupled receptors (11). Both wild type and K268R mutant receptors were capable of this interaction with GFP-β-arrestin2 as shown in Fig. 4b. According to previous reports β-arrestin receptor interaction requires phosphorylation of serine clusters in the V2R tail region (4, 14). Because the K268R mutant receptor is capable of recruiting β-arrestin2, its phosphorylation state is not likely to be affected. Moreover, the nonubiquitinated V2R is internalized similar to the wild type receptor. As shown in Fig. 4c, ~80% of the surface receptors (K268R and the wild type) are internalized after 60 min. Therefore, lack of ubiquitination in K268R had no effect on receptor internalization.

To determine whether ubiquitination could regulate the life cycle of V2R, the half-lives of the wild type and the mutant K268R receptor were determined by pulse-chase experiments.
In unstimulated cells, wild type and the K268R V2 receptors were then immunoprecipitated, resolved on gels, and analyzed. With 35S-labeled Met and 35S-labeled Cys, tagged receptors were depleted in agonist-stimulated cells. Our data demonstrate a striking difference in the pattern of degradation for the receptors. The delay in K268R degradation reflects a possible lag in receptor sorting following stimulation. The alternate pathway of degradation for the nonubiquitinated receptor leads to elevated levels of receptor for up to 3 h. The agonist-dependent degradation of the K268R receptor mutant appears to be linear, whereas agonist-dependent degradation of the wild type receptor is non-linear and asymptotic. However, if one looks at the data presented in Fig. 5c, it becomes apparent that there is no significant difference in the half-life of the mutant receptor in the presence or absence of agonist. This suggests that the difference in the shape of the time curves for the mutant receptor degradation in the presence or absence of agonist may be more apparent than real.

Our data suggest that two different pathways degrade V2R. A slow degradative pathway in unstimulated cells is responsible for maintaining a balance between the synthesis and destruction of the V2 receptor. This slow pathway continuously degrades the V2 receptor using a ubiquitin-independent mechanism, because the mutant K268R that does not get ubiquitinated is still degraded by this pathway. Following agonist stimulation and subsequent ubiquitination of the receptor, a rapid ubiquitin-dependent mechanism speeds up the receptor degradation. Because of the lack of ubiquitination, the K268R mutant receptor degrades at a slower rate in the presence of agonist stimulation (Fig. 5c).

It has been reported that in-frame fusion of a single ubiquitin moiety to an otherwise stable plasma membrane ATPase protein in yeast leads to rapid endocytosis and vacuolar degradation of the chimeric protein (14). Furthermore, recent studies implicate postendocytic sorting to be mediated via ubiquitin-dependent mechanisms via the yeast Vps proteins and their mammalian homologs such as TSG101 and Hrs (15, 16). Such recognition signals are single ubiquitin moieties on the endocytic cargo and the sorting proteins that are involved, such as Eps15 (17). On the other hand, the cell surface protein Gap1 (general amino acid permease) becomes polyubiquitinated before being degraded in the yeast vacuole (18).

In general, variety in the number of ubiquitin moieties that could be covalently attached to a protein can modulate the trafficking signal generated by this post-translational modification. For many proteins, single ubiquitin attachment signals internalization, whereas polyubiquitination tags them for destruction. In the case of V2R, the presence of an ubiquitinated receptor as a smear on Western blots is indicative of receptor polyubiquitination, and our studies of wild type and K268R receptor half-lives confirm that ubiquitination is the signal for rapid receptor degradation.

Our data are consistent with a number of mechanisms for V2 receptor degradation. Previous studies have suggested that V2 receptor can be degraded by processes that involve lysosomes, proteasomes, and cell membrane-bound metalloproteinases (19, 22). The rapid agonist-stimulated receptor degradation likely involves lysosomes, because like many seven-transmembrane-spanning receptors, it has been demonstrated that V2 receptor traffics to lysosomes upon agonist stimulation (19). Our data, however, indicate that polyubiquitination of the receptor and the proteasomal machinery are also likely to be involved in the agonist-stimulated degradation because the proteasomal inhibitor MG132 is necessary to observe V2 receptor ubiquitination. Proteasomal involvement in endosomal sorting to the lysosomes has been described for several plasma membrane proteins (19–21). On the other hand, it is possible that the slow constitutive ubiquitin-independent degradation of the receptors might be carried out by other mechanisms such as the previously described cell membrane-bound metalloproteinases (22).

Acknowledgments—We thank Dr. Wei Chen for assistance with confocal microscopy, Chris D. Nelson for helpful discussion, and Donna Addison and Julie Turnbough for excellent secretarial assistance.

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J. Biol. Chem. 2003, 278:45954-45959.
doi: 10.1074/jbc.M308285200 originally published online September 5, 2003

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