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KLHL12-mediated ubiquitination of the dopamine D4 receptor does not target the receptor for degradation

Pieter Rondou [a,1], Kamila Skieterska [a], Ann Packeu [b], Béatrice Lintermans [a], Peter Vanhoenacker [a,2], Georges Vauquelin [b], Guy Haegeman [a], Kathleen Van Craenenbroeck [a,*]

[a] Laboratory of Eukaryotic Gene Expression and Signal Transduction (LEGEST), Department of Physiology, Ghent University-UGent, K.L. Ledeganckstraat 35, B-9000 Gent, Belgium
[b] Institute for Molecular Biology and Biotechnology, Free University Brussels-VUB, Pleinlaan 2, B-1050 Brussel, Belgium

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ABSTRACT

In previous studies, we identified KLHL12 as a novel interaction partner of the dopamine D4 receptor that functions as an adaptor in a Cullin3-based E3 ubiquitin ligase complex to target the receptor for ubiquitination. In this study, we show that KLHL12 promotes poly-ubiquitination of the receptor by performing ubiquitination assays in eukaryotic cells. Furthermore, we demonstrate that KLHL12 not only interacts with both immature, ER-associated and mature, plasma membrane-associated D4 receptors, but also promotes ubiquitination of both receptor subpools. Unexpectedly, however, KLHL12-mediated receptor ubiquitination does not promote proteasomal degradation of newly synthesized receptors through the ER-associated degradation pathway or lysosomal degradation of mature receptors. Moreover, our data reveal that D4 receptors do not undergo agonist-promoted ubiquitination or degradation, in contrast to many other G-protein-coupled receptors (GPCRs) indicating that ubiquitination of GPCRs does not defaultly lead to receptor degradation. Interestingly, KLHL12 does also interact with β-arrestin2 but this has no effect on the ubiquitination or localization of β-arrestin2 nor on the internalization of the D4 receptor.

1. Introduction

Dopamine is a neurotransmitter that plays an important role in various processes in the mammalian brain, such as reward, motivation, learning, memory, movement, and endocrine regulation, by binding to specific receptors on target cells [1,2]. Dopamine receptors belong to the GPCR (G-protein-coupled receptor) superfamily, and can be subdivided into D1-like (D1, D5) receptors that activate adenylyl cyclase through coupling with Gs-proteins, and D2-like (D2, D3, D4) receptors that inhibit adenylyl cyclase via Gi/o-proteins. The D4 receptor contains an important polymorphism in its third intracellular loop, consisting of a variable number of tandem repeats (VNTR), resulting in different possible receptor variants (from D4.2 to D4.11) [3–5]. Interestingly, 7-repeat alleles have been associated with ADHD, demonstrating the importance of this unique polymorphism [6].

Ubiquitination is a post-translational modification that involves the covalent attachment of ubiquitin (76 aa polypeptide) on lysine residues of substrate proteins: first, the ubiquitin-activating enzyme (E1) activates ubiquitin, which is then transferred to the ubiquitin-conjugating enzyme (E2). Finally, ubiquitin is attached to the substrate by (E3) ubiquitin ligases, which are multiprotein complexes that provide specificity towards the substrate [7]. Attachment of a single ubiquitin moiety to a single residue of the substrate results in mono-ubiquitination. When several lysine residues undergo mono-ubiquitination, this results in multi-ubiquitination. Moreover, ubiquitin itself contains seven lysine (K) residues, which can also serve as acceptor sites for additional ubiquitin moieties, resulting in poly-ubiquitin chains. Although all these ubiquitin residues were demonstrated to be able to form poly-ubiquitin chains, K48- and K63-linked chains are the most abundant [8,9]. These different types of ubiquitination can result in different possible outcomes for ubiquitinated substrates. The most frequent type, K48-linked poly-ubiquitination, targets substrate proteins for proteasomal degradation [10,11]. Besides proteasomal degradation, the second major degradative pathway in the cell is based on lysosomes. In contrast to K48-linked poly-ubiquitination, K63-linked poly-ubiquitination does not target substrates for proteasomal degradation, but rather plays a role in processes such as endocytic trafficking, inflammation, protein

Abbreviations: GPCR, G-protein-coupled receptor; BTB, broad-complex, tramtrack, and Bric à Brac; HEK293, Human Embryonic Kidney 293; CHO, Chinese hamster ovary; IP, immunoprecipitation; IB, immunoblotting; E3, ubiquitin-conjugating enzyme; HA, hemagglutinin; siRNA, small interfering RNA; ER, endoplasmic reticulum; ERAD, ER-associated degradation; PM, plasma membrane; BFA, brefeldin A; wt, wild-type; Cul3, Cullin 3; Ub, ubiquitin; MW, molecular weight.

* Corresponding author. Laboratory of Eukaryotic Gene Expression and Signal Transduction (LEGEST), Ghent University-UGent, K.L. Ledeganckstraat 35, B-9000 Gent, Belgium. Tel.: +32 9/264 51 35; fax: +32 9/264 53 04.
E-mail address: Kathleen.VanCraenenbroeck@UGent.be (K. Van Craenenbroeck).
[1] Present address: Center for Medical Genetics Ghent (CMGG), Ghent University Hospital — UZ Gent, De Pintelaan 185, B-9000 Gent, Belgium.
[2] Present address: ActoGeniX, Technologiepark 4, B-9052 Zwijnaarde (Gent), Belgium.

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translation, activation of certain protein kinases and DNA repair [12,13]. Similarly, mono-ubiquitination does not lead to proteasomal degradation, but functions in membrane trafficking, regulation of the endocytic machinery and lysosomal degradation, viral budding, and DNA repair [14–17]. In all these different cellular processes, ubiquitin serves as a recognition site for specific and selective recruitment of different ubiquitin-binding domains (UBDs). For example, K48-linked poly-ubiquitin chains have a more dense conformation, whereas K63-linked poly-ubiquitin chains are more extended, and hence, both can be recognized by a different specific subset of UBDs [18–20].

Ubiquitin also plays a role in GPCR regulation. A major type of GPCR ubiquitination is independent of agonist stimulation. For some GPCRs, ubiquitin-dependent proteasomal degradation leads to basal turnover of cell surface receptors [21,22]. Additionally, proteasomal degradation through the ER-associated degradation (ERAD) pathway [23] is involved in the regulation of many GPCRs, such as the dopamine D4 receptor [24]. This quality control mechanism promotes newly synthesized but misfolded proteins to undergo ubiquitination in the ER, targeting them for proteasomal degradation [25].

Another major type of GPCR ubiquitination occurs upon agonist induction and can play a role in receptor internalization [26–28]. For the prototypic β2-adrenergic receptor, and for other GPCRs (CXCR4, V2 vasopressin receptor), it was demonstrated that agonist-dependent receptor ubiquitination is not required for receptor internalization, but rather functions to target internalized receptors to lysosomes for degradation [29–31]. However, exceptions exist; the β1-adrenergic receptor, for example, is resistant to agonist-induced ubiquitination and agonist-promoted degradation [32]. Another exception is the delta opioid receptor (δOR), which undergoes agonist-induced lysosomal degradation but does not seem to require ubiquitination for this process [33,34]. A hallmark for GPCR internalization is often the recruitment of β-arrestins [35,36], and agonist-stimulated ubiquitination of GPCRs and of β-arrestins can play a role in receptor internalization/degradation [29,37,38]. Recently, we have shown that the D4 receptor is resistant to agonist-induced receptor phosphorylation, β-arrestin1/2-recruitment, degradation and internalization [39]. Here we show that receptor stimulation neither induces ubiquitination of the receptor nor of β-arrestin2.

In our previous study, we identified the BTB-Kelch protein KLHL12 as a novel D4 receptor-interacting partner that specifically binds to the VNTR polymorphic region of this receptor [40]. Furthermore, we demonstrated that KLHL12 functions as an adaptor in a Cullin3-based E3 ubiquitin ligase complex that specifically promotes ubiquitination of the D4 receptor. In this study, we wanted to characterize and unravel the function of KLHL12-mediated receptor ubiquitination. We provide evidence that KLHL12 interacts with and promotes ubiquitination of both immature, ER-associated and mature, plasma membrane-associated D4 receptors. However, KLHL12-mediated receptor ubiquitination does not lead to receptor degradation, which is highly remarkable and most interesting in view of the current literature assigning a role for GPCR ubiquitination in targeting receptors for degradation.

We further demonstrate that KLHL12 can interact with β-arrestin2, although this has no effect on ubiquitination or localization of β-arrestin2 or on D4 receptor internalization, upon agonist induction.

2. Materials and methods

2.1. Plasmids and antibodies

Plasmids encoding the HA D4.2 receptor, FLAG D4.4 receptor, HA D2 receptor, Etag KLHL12, and FLAG Ub, were described before [39,40]. The ubiquitin constructs coding for wild-type (wt) HA Ub, the single mutants HA K29R, HA K48R and HA K63R, and the triple mutant FLAG Ub K29,48,63R were kind gifts from Dr. Dikič (Goethe Universität, Frankfurt, Germany), and the wt cmyc Ub was obtained from Dr. Kopito (Stanford University, Stanford, CA). β-arrestin2 GFP, HA β2-adrenergic receptor and FLAG β-arrestin2 were kind gifts from Dr. R. Lefkowitz (Duke University, Durham, NC).

The D4.2 receptor contains four lysine (K) residues in its primary structure, namely at sites 229 (in the third intracellular loop before the 'variable number of tandem repeat' polymorphism), 304, 311 (both in the third intracellular after the polymorphism) and 381 (in the C-terminal tail). The HA D4.2 receptor construct was used as a template for the application of single-nucleotide mutations (encoding arginine instead of lysine) using the QuikChange® Site-Directed Mutagenesis Kit from Stratagene (La Jolla, CA, USA). The quadruple mutant D4.2 K229,304,311,381R is further denoted as 4 K➔R.

Primary antibodies used were mouse monoclonal anti-HA (clone 16B12; Covance Research Products, Berkeley, CA), mouse monoclonal anti-Etag (Amersham Biosciences, Roosendaal, The Netherlands), rabbit anti-Etag (GenScript), rabbit anti-c-myc (Sigma), rabbit anti-HA (GeneTex), horseradish peroxidase (HRP)-conjugated mouse monoclonal anti-Etag (Amersham Biosciences), mouse monoclonal anti-ubiquitin clone P4D1 (Cell Signaling, Danvers, MA), mouse monoclonal anti-ubiquitin clone FK1 (Biomol, Plymouth Meeting, PA), mouse monoclonal anti-poly-ubiquitin (K63-linkage specific) clone HWA4C4 (Biomol), mouse monoclonal anti-FLAG M2 (Sigma, St. Louis, MO) (immunoprecipitation), HRP-conjugated mouse monoclonal anti-FLAG M2 (Sigma) (immunodetection in HEK293T cells), mouse monoclonal anti-FLAG M1 (Sigma) (immunodetection in stably transfected CHO FLAG D4.4 cells), mouse monoclonal anti-actin (Sigma), and rabbit polyclonal anti-β-catenin (Abcam, Cambridge, MA). Secondary antibodies used were HRP-conjugated anti-mouse and anti-rabbit IgG (Cell Signaling) and Alexa-350-conjugated and Alexa-594-conjugated anti-mouse and anti-rabbit IgG (Invitrogen).

2.2. Chemical products

Brefeldin A (BFA), the lysosomal inhibitor chloroquine, and the agonists dopamine and quinpirole were purchased from Sigma; the proteasomal inhibitor MG-132 and the D4 receptor antagonist L745-870 were from Calbiochem (Merck; Darmstadt, Germany) and Tocris (Bristol, UK), respectively.

2.3. Cell culture and transfection

HEK293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen), supplemented with 10% fetal calf serum, penicillin (100 U/ml), and streptomycin (100 μg/ml) in a controlled environment (37 °C, 98% humidity, 5% CO_2). HEK293T cells were transfected using the Polyethylenimine (PEI) method. Therefore, cells were grown in 10 cm dishes until subconfluency. The medium was refreshed with 9 ml DMEM, supplemented with 2% fetal calf serum, 1 h before transfection. A mixture of 475 μl serum-free medium and 25 μl (1 μg/μl) PEI was added dropwise to a solution of 500 μl serum-free medium containing 10 μg DNA. Upon mixing thoroughly and incubation for 10 min at room temperature, the DNA/PEI mixture was added dropwise to the cells. 6 h later, the medium was refreshed with DMEM, supplemented with 10% fetal calf serum. CHO FLAG D4.4 cells were cultured in αMEM (Gibco, Invitrogen) supplemented with 10% fetal calf serum, penicillin (100 U/ml), streptomycin (100 μg/ml), and 0.5 mg/ml G418 (Geneticin, Gibco) in a controlled environment (37 °C, 98% humidity, 5% CO_2). CHO FLAG D4.4 cells were transfected using Lipofectamine™ (Invitrogen) (4.5 μl/μg DNA), according to the manufacturer's guidelines. The amount of plasmid DNA used for transfection is indicated for each experiment.

2.4. Immunoblot analysis and co-immunoprecipitation assays

After transfection, cells were washed twice with ice-cold phosphate-buffered saline, harvested and the cell pellet was frozen at