Regulation of Endocytic Trafficking of Receptors and Transporters by Ubiquitination: Possible Role in Neurodegenerative Disease

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Abstract Ubiquitination has recently emerged as the major regulatory mechanism of endocytic trafficking of transmembrane proteins. Ubiquitin-controlled trafficking and endocytosis regulate the function of various receptors, channels and transporters in neurons, and deregulation of the ubiquitination system is associated with neurodegenerative diseases. Hence, we will focus on recent advances in understanding the mechanisms and functional roles of ubiquitination of two families of transmembrane proteins: (1) receptor tyrosine kinases, using the receptor for epidermal growth factor (EGFR) as a prototypic member of the family; and (2) monoamine transporters, using an example of the plasma membrane dopamine transporter (DAT). Both these families of receptors and transporters are intimately involved in brain development, regulation of survival signaling in adult neurons, neurotransmission, neuronal cytotoxicity and neurodegeneration. Endocytosis regulates the duration and intensity of the EGFR signaling. Endocytosis of DAT controls the re-uptake of dopamine in dopaminergic neurons, thus regulating dopamine neurotransmission in the brain. Our recent studies revealed unexpected similarities in the regulation of endocytosis of these two structurally distinct families of proteins by ubiquitination. We have mapped ubiquitin conjugation sites in the EGFR and demonstrated that mutation of these sites results in inhibition of the lysosomal targeting and degradation of EGFR. However, EGFR ubiquitination appears not to be essential for the internalization step of the EGFR trafficking. Surprisingly, we have recently found that DAT is also ubiquitinated and the extent of its ubiquitination is dramatically increased upon activation of protein kinase C (PKC). The ubiquitination sites in DAT were also mapped by mass spectrometry. Mutations of a cluster of three lysines in the N-terminal tail of DAT blocked the clathrin-mediated endocytosis of DAT. Screening of the library of small interfering RNAs revealed that NEDD4-2 is an E3 ubiquitin ligase responsible for ubiquitination of DAT and necessary for PKC-dependent DAT endocytosis. Thus, our studies revealed that both EGFR and DAT are ubiquitinated

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at the plasma membranes and endosomes, and this ubiquitination regulates their turnover and subcellular localization. Interestingly, both EGFR and DAT are modified by Lys63-linked poly-ubiquitin chains. We hypothesize that short, Lys63-linked chains are the major ubiquitin-based molecular signals operating during endocytic trafficking in mammalian cells.

1 Introduction

The activities of neuronal cells and their survival are controlled by various receptor, channel and transporter proteins present at the surface of these neurons, where they interact with their ligands and substrates. Various classes of transport proteins essential for synaptic transmission and neuronal signaling function in the intracellular compartments, such as synaptic vesicles and endosomes. For example, receptor tyrosine kinases (RTKs), such as TrkA receptors for the nerve growth factor, require endocytosis at the distal axonal processes and an axonal transport of TrkA signaling complexes in endosomes for the retrograde survival signaling in the neuronal soma (Zweifel et al. 2005). Endocytosis of APP appears to be necessary for the neuronal activity-dependent extracellular accumulation of the amyloid-β peptide (Cirrito et al. 2008). Thus, aberrant endocytic trafficking leading to mis-localization of transmembrane proteins within the neuronal cell often underlies the mechanisms responsible for the development of the neurodegenerative disease.

Rapid and dynamic regulation of the amounts of receptors and transport proteins at the plasma membrane and intracellular membrane compartments in the synapse and extrasynaptically is achieved by means of selective endocytosis and recycling of these proteins. Many receptors and transport proteins are rapidly endocytosed in a constitutive or stimuli-dependent manner. Subsequently, the internalized transmembrane proteins (i.e., cargo) are either recycled back from endosomes to the plasma membrane, or accumulate in specialized compartments, such as synaptic vesicles and endosomes, or are sorted to lysosomes for degradation. The mechanisms of endocytosis and post-endocytic trafficking of membrane proteins have been extensively studied over the last 30 years; however, molecular details of many steps of these processes remain poorly understood.

Posttranslational modification of transmembrane proteins by the covalent attachment of ubiquitin has recently emerged as the major regulatory mechanism of endocytic trafficking of these proteins. Many of the original observations of ubiquitination of the endocytic cargo and regulation of endocytosis by ubiquitination were made in yeast (Hicke and Riezman 1996; Kolling and Hollenberg 1994). Among mammalian ubiquitinated cargo are RTKs; Notch and its transmembrane ligands, cytokine and interferon receptors; various channels and transporters; G protein coupled receptors (GPCR); and other types of transmembrane proteins (Hicke and Dunn 2003; Staub and Rotin 2006). Our laboratory is focusing on the mechanisms and functional roles of ubiquitination of two classes of molecules: (1) RTKs, using a prototypic member of the family, the epidermal growth factor (EGF) receptor (EGFR)

as an experimental model; and (2) plasma membrane solute transporters, using the plasma membrane dopamine transporter (DAT) as an experimental model.

2 Modification of Proteins by Ubiquitin

Ubiquitination is a posttranslational modification that mediates the covalent conjugation of ubiquitin, a highly conserved protein of 76 amino acids, to protein substrates. Ubiquitination was originally thought to target proteins for degradation by the 26*S* proteasome (Hershko and Ciechanover 1992). However, the role of ubiquitination in many non-proteosomal processes in the cell, including membrane trafficking, DNA repair, and transcription, has been recently revealed (Mukhopadhyay and Riezman 2007; Pickart and Fushman 2004). The observations of an abnormal enrichment of inclusion bodies with ubiquitin in Huntington's disease and many other neurodegenerative disorders, including Alzheimer's and Parkinson's diseases (Lowe et al. 1988; Mayer et al. 1989), have suggested that dysfunction in ubiquitin metabolism may contribute to the pathogenesis of these diseases (DiFiglia et al. 1997; Ross and Pickart 2004).

The mechanism of ubiquitination involves the sequential action of several enzymes. In the initial step, the E1 ubiquitin-activating enzyme forms a thioester bond between its catalytic cysteine and the carboxyl group of Gly76 of ubiquitin in an ATP-dependent manner. The ubiquitin molecule is then transferred to an E2 ubiquitin-conjugating enzyme, which also forms a thioester bond between its cysteine and ubiquitin. Finally, ubiquitin is transferred to a lysine residue of the substrate with the help of an E3 ubiquitin ligase. The family of isopeptidases responsible for the removal of ubiquitin from the substrate is called deubiquitination enzymes (DUBs; Millard and Wood 2006).

Attachment of a single ubiquitin moiety to a single lysine on a substrate results in monoubiquitination (Fig. 1). Monoubiquitin can be conjugated to several lysine residues on the same substrate molecule, resulting in multi-monoubiquitination. Additional ubiquitin molecules can be attached to the lysine residues in ubiquitin itself, leading to the formation of di-ubiquitin and polyubiquitin chains conjugated to a single lysine of the substrate. Although ubiquitin contains seven lysine residues, all capable of conjugating ubiquitin, Lys48- and Lys63-linked chains are the most abundant. The majority of published studies suggest that Lys48-linked chains serve as the recognition signal by the proteasome and target proteins for proteasomal degradation (Pickart and Fushman 2004). In contrast, Lys63-linked ubiquitin chains do not target proteins to proteasome but mediate interactions with protein machineries involved in endocytic trafficking, inflammatory response, protein translation, and DNA repair (Pickart and Fushman 2004). Similarly, it is widely accepted that monoubiquitination does not target proteins to the proteasome but serves as a molecular recognition signal in membrane trafficking, regulation of endocytic machinery, and possibly other cellular processes (Staub and Rotin 2006). Interestingly, the impairment of the ubiquitin-mediated protein degradation and proteosomal function

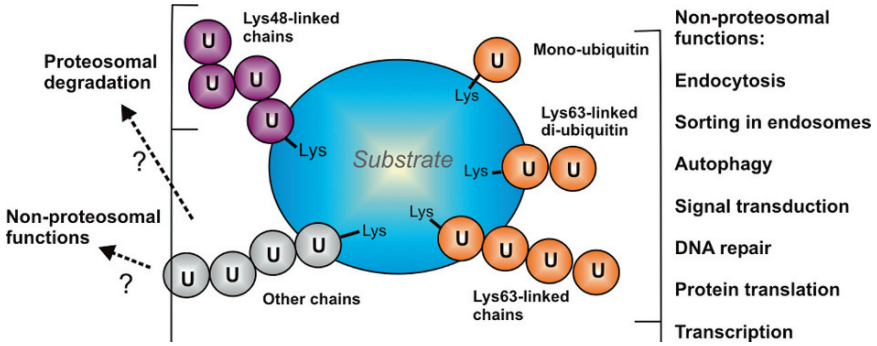

Fig. 1 Types of ubiquitin conjugation. The last residue of ubiquitin (Gly76) is covalently attached to the ε-amino group of lysines in the substrate. Substrates can be modified with a single ubiquitin molecule at single (monoubiquitination) or multiple (multi-monoubiquitination) lysine residues. Further ubiquitin conjugation to the lysine residues of the ubiquitin molecule results in the attachment of di-ubiquitin to the substrate or a substrate polyubiquitination. The main functions of monoubiquitination and the most frequently detected ubiquitin chains linked through Lys63 or Lys48 of ubiquitin are listed. Lys48- or Lys63-linked chains are shown in a "closed" or "extended" conformation, respectively, resulting in different mechanisms of recognition of these chains by ubiquitin binding domains (UBDs). Ubiquitin chains linked to other lysines of the ubiquitin have been implicated in the proteosomal and non-proteosomal processes

in neurodegenerative diseases leads to the accumulation of proteins containing mainly Lys48-linked polyubiquitin chains but also Lys63- and Lys11-linked chains (Bennett et al. 2007).

All functions of ubiquitin are accomplished through specific interactions of the ubiquitin moiety with the ubiquitin-binding domains (UBDs) found in many proteins (Hicke et al. 2005). All of the helical UBDs interact with hydrophobic Ile44 in ubiquitin, although there are several types of UBD that have different modes of recognition of mono- and poly-ubiquitin (Hurley et al. 2006). Structural studies demonstrated that Lys48-linked di-ubiquitin has a closed conformation, whereas Lys63-linked di-ubiquitin has an extended conformation, thus implying their selective recognition by different types of UBDs (Raasi et al. 2005; Varadan et al. 2004, 2005).

3 Regulation of Endocytosis of EGFR by Ubiquitination

EGFR regulates growth and survival signaling in many types of cells. EGFR signaling via the Akt pathway plays a key role in the protection of dopaminergic neurons from neurodegeneration in Parkinson's disease (Inoue et al. 2007; Iwakura et al. 2005). Binding of EGF or other ligands to the surface EGFR leads to activation of the receptor kinase and phosphorylation of C-terminal tyrosine residues, which results in recruitment of adaptor proteins and enzymes to the receptor and initiation of several signaling cascades. Activation of EGFR also causes rapid internalization

of ligand-occupied EGFR through clathrin-coated pits into endosomes and subsequent efficient sorting of these complexes to the lysosome degradation pathway. Endocytosis of EGFR has a key role in the control of the intensity and duration of signaling by the receptors by down-regulating the activated EGFRs. Endocytosis is also orchestrating signaling processes by localizing EGFR and down-stream signaling effectors to various intracellular compartments. However, the molecular mechanisms of endocytosis and post-endocytic sorting of EGFR and other RTKs remain elusive.

The first clue to the mechanism of EGFR internalization came from RNA interference (RNAi) experiments in which siRNA knock-down of the GrbB2 adaptor protein demonstrated that this protein is essential for the clathrin-mediated endocytosis of EGFR. Dominant-negative mutants of Grb2 and mutation of Grb2 binding sites in EGFR reduced the internalization of EGFR. Grb2 was present in clathrin-coated pits in EGF-stimulated cells. All this evidence strongly indicated that Grb2 is important for the internalization of EGFR.

Grb2 binds to EGFR via its SH2 domain and functions as a link to bring to the receptor other proteins that are associated with the SH3 domains of Grb2 (Fig. 2). One family of proteins called *Cbl*s that interact with Grb2 has been previously implicated in EGFR endocytosis and degradation, and we therefore tested the importance of Grb2-Cbl interaction in EGFR internalization. The human Cbl family of proteins consists of three isoforms, c-Cbl, Cbl-b and Cbl-c (Thien and Langdon 2001). Cbls are the E3 ubiquitin ligases. All three Cbls have an N-terminal tyrosine kinase binding (TKB) domain connected (with a linker segment) to a RING finger domain. c-Cbl and Cbl-b each have an extended C-terminal tail containing proline-rich motifs capable of binding to SH3 domains. The TKB domain directly binds to the specific phosphotyrosine-containing motifs in EGFR and other RTKs. The RING domain of the E3 ubiquitin ligase recruits an E2 enzyme and positions it so that the ubiquitin moiety can be transferred from E2 to the substrate. In our experiments, mutants of Cbl lacking Grb2 binding sites or RING domain activity have imposed a dominant-negative effect on EGFR internalization, suggesting the role of Cbl and its functional domains in EGFR internalization. This hypothesis was supported in experiments where knockdown of two Cbls (c-Cbl and Cbl-b) that interact with Grb2 by siRNA blocked internalization of EGFR.

Our studies using FRET demonstrated that the Grb2-Cbl complex is recruited to activated EGFR. The TKB domain of Cbl also directly binds to the receptor phosphorylated Tyr1045. Both direct and Grb2-mediated interactions of Cbl with the EGFR are necessary for the full ubiquitination of EGFR (Huang and Sorkin 2005; Jiang and Sorkin 2003; Levkowitz et al. 1999). This putative mechanism of dual Cbl interaction with an RTK was also demonstrated for another RTK, HGF/c-Met receptors (Peschard et al. 2001). Mutation of Tyr1045 did not affect EGFR internalization, suggesting that the direct interaction of Cbl with EGFR and full ubiquitination of the receptor are not necessary for internalization. Because the Y1045A mutant of EGFR still has residual (10–20%) ubiquitination, the question was whether this minor ubiquitination mediates internalization of EGFR.

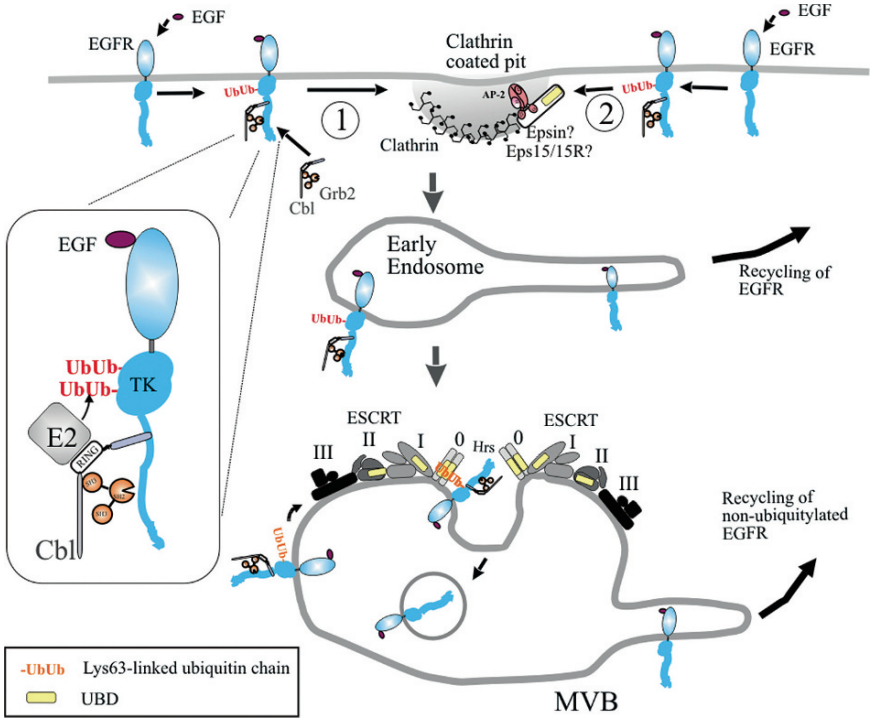

Fig. 2 Interactions of the EGF receptor leading to receptor ubiquitination and the hypothetic model of EGFR endocytosis. EGF binding activates the receptor tyrosine kinase and results in the phosphorylation of Tyr1045, Tyr1068, and Tyr1086 in the C terminus of EGFR. The SH3 domains of Grb2 are associated with the C-terminus of c-Cbl or Cbl-b. A Grb2-Cbl complex binds to the receptor by means of the interaction of the SH2 domain of Grb2 with phosphorylated Tyr1068 or Tyr1086, and the interaction of the tyrosine kinase binding (TKB) domain of c-Cbl/Cbl-b with phosphoTyr1045. Recruitment of E2 enzymes to the RING domain of Cbl results in the covalent attachment of mono-ubiquitin and poly-ubiquitin chains to the kinase domain of the receptor. EGFR is internalized via clathrin-coated pits with participation of Grb2 and Cbl by an unknown mechanism (1) or by means of the interaction of ubiquitin attached to the receptor kinase domain with the proteins containing UBD domains and located in coated pits (Eps15/Eps15R/epsin). The latter proteins can interact with the AP-2 complex or directly with clathrin. After fusion of clathrin-coated vesicles with early endosomes, EGFR can either recycle directly back to the plasma membrane or remain in the maturing endosome that acquires ESCRT complexes. Ubiquitinated receptors bind to the UBD of the ESCRT-0 complex (HRS) and eventually become trapped in the intralumenal vesicles of MVB. Non-ubiquitinated receptors can recycle back to the cell surface through the tubular extensions of MVB

To directly address the role of EGFR ubiquitination, we used mass-spectrometry analysis to map ubiquitination sites in the EGFR. Surprisingly, this analysis revealed that all the major sites of EGFR ubiquitination were located within the conserved kinase domain of the receptor (Huang et al. 2006). Additionally, in the absence of the major conjugation sites, other lysines became ubiquitinated, suggesting that EGFR ubiquitination sites were highly redundant. Importantly, quantitative

mass-spectrometry analysis showed that EGFRs contained approximately 50% of mono-ubiquitin and 50% of poly-ubiquitin and that the most abundant type of polyubiquitination was the Lys63-linked chains (Huang et al. 2006).

Mutation of the major ubiquitination sites in the EGFR (lysine-to-arginine; KR mutations) had no effect on its internalization (Huang et al. 2006). However, the possibility remained that a residual cryptic ubiquitination of EGFR KR mutants was sufficient for their internalization. Therefore, in recent studies a number of other lysine residues in the EGFR kinase domain were mutated. Some lysines could not be mutated due to the loss of receptor kinase activity. However, a mutant in which 15 lysines were mutated possessed normal kinase activity but very little if any ubiquitination (about 1% of wild-type EGFR). This mutant was normally internalized, indicating that EGFR ubiquitination was not essential for internalization.

One of the multi-KR mutants, 16KR, displayed a low internalization rate. However, it was found that this mutant had reduced tyrosine kinase activity. Because tyrosine kinase activity is critical for EGFR internalization, reduced activity could explain the low rate of internalization of this mutant. However, when two major ubiquitination sites were added back by mutating two arginines back to lysines (16KR/2RK mutant), the resulting mutant was partially ubiquitinated and internalized at a rate comparable to wild-type EGFR, despite its partially reduced kinase activity. These data suggested that ubiquitination of the receptor might mediate its internalization even in the absence of the full kinase activity. Altogether, the EGFR mutagenesis experiments suggested that there were at least two redundant mechanisms of EGFR internalization through clathrin pathway. One mechanism required a full kinase activity of the receptor but did not require ubiquitination. Another mechanism utilized ubiquitination of the receptor.

4 Role of Ubiquitination in the Endosomal Sorting of EGFR

After internalization into early endosomes, receptors are either recycled back to the plasma membrane or sorted to late endosomes and lysosomes (Fig. 2). After 15–20 min of continuous EGF-induced endocytosis, EGF and EGFR accumulate in the intralumenal vesicles of multi-vesicular endosomes or bodies (MVBs) that are mostly located in the perinuclear area of the cell (McKanna et al. 1979; Miller et al. 1986). EGFRs that are incorporated into intralumenal vesicles cannot recycle. MVBs have tubular membrane extensions that are thought to be responsible for recycling of receptors not incorporated into internal vesicles (Hopkins 1992).

When the degradation rates of ubiquitination-deficient EGFR mutants were analyzed, it was found that receptor degradation was significantly decreased in all mutants of EGFR in which ubiquitination was reduced (Huang et al. 2006). Moreover, fluorescence microscopy analysis demonstrated that these mutants were inefficiently delivered to late endosomes. Finally, preliminary electron microscopy studies showed that ubiquitin-deficient EGFR mutants accumulated at the limiting membrane of MVB and in recycling endosomes whereas their incorporation into

intralumenal vesicles of MVBs was significantly reduced as compared to wild-type EGFR. Therefore, ubiquitination is critical for the efficient sorting of EGFR in MVB and lysosomal targeting of the receptor.

These studies support the model whereby the ubiquitinated EGFR in endosomes interacts with the UBD of the hepatocyte growth factor receptor phosphorylation substrate (Hrs) that is associated with another UBD-containing protein, STAM1/2 (ESCRT, endosomal sorting complex required for transport, −0 complex; Bache et al. 2003; Hurley and Emr 2006). It is hypothesized that multiprotein ESCRT-I, II and III complexes surrounding cargo associated with ESCRT-0 then generate inward invagination of the limiting membrane of MVBs, thus capturing EGFR in the forming intralumenal vesicle (Babst et al. 2000; Bache et al. 2006; Bowers et al. 2006; Hurley and Emr 2006; Slagsvold et al. 2006).

Degradation of EGF and the EGFR is completely blocked by lysosomal inhibitors, suggesting that it occurs in lysosomes (Carpenter and Cohen 1976; Stoscheck and Carpenter 1984). Although the use of proteasomal inhibitors can also reduce EGFR degradation (Longva et al. 2002), these inhibitors may affect the activity of lysosomal enzymes and turnover of ESCRT proteins, or reduce the ubiquitin pool in the cell. Therefore, the effects of proteasomal inhibitors on EGFR degradation are likely indirect. The current model suggests that proteolytic enzymes are delivered to MVBs through fusion with "primary" lysosomal vesicles, which leads to the formation of mature lysosomes and proteolysis of the intralumenal content of these organelles (Miller et al. 1986).

A number of proteins have been proposed to modulate the process of EGFR targeting to the lysosome degradation pathway, mainly through affecting Cbl and Cbl-mediated ubiquitination of EGFR. Interestingly, EGFR degradation is regulated by the protein called Spartin, which is mutated in Troyer syndrome, an autosomal recessive hereditary spastic paraplegia. Thus, impaired endocytosis of EGFR or similar RTKs may underlie the pathogenesis of Troyer syndrome.

Importantly, regulation of the endocytic trafficking and stability (turnover rates) by ubiquitination is a common feature of several families of RTKs, including RTKs that are critical for the neuronal development and the survival signaling in adult neurons. For example, ubiquitination of the receptor for the nerve growth factor, TrkA, has been recently reported and implicated in the regulation of TrkA endocytosis (Arevalo et al. 2006; Geetha et al. 2005). There is disagreement as to what E3 ubiquitin ligase is involved. One study proposed that TrkA is ubiquitinated by the TRAF6 ubiquitin ligase and that this process requires the interaction of TrkA with the $p75^{NTR}$ co-receptor (Geetha et al. 2005). It is noteworthy that, similar to the EGFR, the TrkA was proposed to be polyubiqutinated by Lys63-linked chains, which was shown to be critical for endocytosis (Geetha et al. 2005). In contrast, another study claimed that TrkA is ubiquitinated by another E3 ligase, termed neuronal precursor cell expressed developmentally downregulated (NEDD4-2), which contains a HECT (homologous to E6-AP C-terminal) domain (Arevalo et al. 2006). Although the data regarding the TrkA-specific ubiquitin ligase are conflicting, both studies suggest that ubiquitination mediates endocytosis of TrkA and therefore affects signal transduction by this RTK. Examples of other RTKs that regulate

survival signaling in the central nervous system and that are regulated by ubiquitination are the platelet-derived growth factor receptor (PDGFR; Mori 1993), ErbB3 and ErbB4 (Cao et al. 2007) and the insulin-like growth factor 1 receptors (Vecchione et al. 2003).

5 Regulation of DAT by Ubiquitination

Plasma membrane neurotransmitter transporters of the SLC6 family play important roles in neuronal cytotoxicity, development of neurodegenerative disorders such as Parkinson's disease, and drug abuse (Gainetdinov and Caron 2003; Gether et al. 2006). Hence, we will focus on our recent studies of one of the members of this family, DAT.

DAT is expressed in dopaminergic neurons, most of which project from the substantia nigra and ventral-tagmental area to the striatum, nucleus accumbens and prefrontal cortex. DAT functions to terminate dopamine (DA) neurotransmission via the reuptake of released DA into dopaminergic neurons. Several psychostimulants and neurotoxins, such as amphetamines, 6-hydroxydopamine (6-OHDA) and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), are transported into the dopamine neuron by DAT, which can lead to dopaminergic neurodegeneration, presumably due to the accumulation of cytosolic dopamine and its oxidation into toxic dopamine-quinones (German et al. 1996; Hanrott et al. 2006; Lotharius and Brundin 2002; Sonsalla et al. 1996; Xu et al. 2005). DAT is shown to directly interact with α-synuclein, a protein involved in the development of Parkinson's disease (Lotharius et al. 2002; Lotharius and Brundin 2002), which results in reduced DAT surface expression (Lee et al. 2001).

DAT has 12 transmembrane domains and intracellular N- and C-termini (Gether et al. 2006). There are no conventional endocytosis sequence motifs in the DAT molecule. RNAi analysis showed that DAT is internalized via a clathrin-mediated pathway (Sorkina et al. 2005). Using HeLa cells expressing human DAT tagged with two epitopes at the N-terminus, we have been able to purify a sufficient amount of DAT protein to perform a mass-spectrometry analysis of purified DAT. This analysis revealed that DAT was constitutively ubiquitinated and that activation of protein kinase C (PKC) substantially increased DAT ubiquitination (Miranda et al. 2005). Furthermore, mass spectrometry also revealed the presence of Lys63-linked polyubiquitin chains in DAT. Interestingly, Western blot analysis of wild-type DAT and various lysine mutants of DAT predicted that each DAT molecule was conjugated at any given time with a single short chain of three ubiquitins.

To examine which proteins regulate PKC-induced endocytosis of DAT, we performed a large-scale RNAi screen using a reverse-transfection library of siRNAs that targeted 53 proteins implicated in endocytosis. This screen revealed that PKC-dependent DAT endocytosis required NEDD4-2 (Sorkina et al. 2006), which is an E3 ubiquitin ligase that has been implicated in the ubiquitination of various transport proteins (Miranda and Sorkin 2007). NEDD4-2 has been most well studied as

an E3 ligase controlling the ubiquitination and endocytosis of ENaC channels (Staub et al. 1996). Furthermore, siRNA to NEDD4-2 dramatically decreased PKC-induced ubiquitination of DAT, suggesting that NEDD4-2 could be an E3 ligase for DAT. The NEDD4 family of proteins has a catalytic C-terminal HECT domain, the N-terminal C2 domain that binds phospholipids in a Ca^{2+}-dependent manner, and two to four WW domains that bind to the PxY (PY) motif (x is any amino acid) in the target protein (Staub and Rotin 2006). Such PY motifs are found in the C-terminal tails of various transmembrane proteins. However, a number of transporters that are regulated by NEDD4-2, including DAT, lack the PY motif. It is possible that NEDD4-2 binds indirectly to DAT, in a manner similar to that described for the IGF-1 receptor (Boehmer et al. 2006). Another possibility is that NEDD4-2 may regulate another E3 ligase that directly ubiquitinates DAT.

PKC-induced DAT ubiquitination takes place initially at the plasma membrane and continues after endocytosis. The major ubiquitination sites in the amino- and carboxyl-termini of DAT were mapped by mass spectrometry (Miranda et al. 2005). Mutagenesis of lysines in the DAT revealed that a cluster of three N-terminal lysines (Lys19, 27 and 35) is essential for PKC-dependent endocytosis of DAT (Miranda et al. 2007). PKC-induced internalization of DAT was dramatically inhibited by mutation of the ubiquitination sites (Miranda et al. 2007).

Finally, an siRNA screen revealed that the PKC-dependent internalization of DAT required the adaptor proteins epsin, Eps15, and Eps15R, which are located in clathrin-coated pits and possess UBDs (Fig. 3; Sorkina et al. 2006). Similarly, epsin and Eps15 have been recently shown to be involved in the NEDD4-2 dependent internalization of ENaC (Wang et al. 2006).

The existing methods of measuring the rate parameters of endocytic trafficking of DAT do not allow the quantification of internalization rates without the contribution of recycling. Therefore, the steps of endocytic trafficking of transporters that are regulated by ubiquitination cannot be precisely defined. Whereas several sets of data suggest that activation of PKC results in the accelerated internalization of DAT in a ubiquitin-dependent manner, it also leads to the accelerated degradation of DAT in lysosomes (Daniels and Amara 1999; Miranda et al. 2005). Therefore, it is likely that DAT ubiquitination also mediates the sorting of DAT to the degradation versus recycling pathway. As described above for the EGFR model (Fig. 2), this sorting probably involves incorporation of the transporters in the intralumenal vesicles of MVB. The observations of the co-localization of DAT with HRS in endosomes (Miranda et al. 2005; Sorkina et al. 2003) and the detection of DAT inside MVBs in DA neurons support this hypothesis (Hersch et al. 1997). It is likely that lysosomal sorting of DAT occurs mainly in the somatodendritic compartment of the dopaminergic neurons where MVBs and lysosomes are easily detected, whereas endocytic trafficking of DAT at the axonal processes in the striatum could be limited by cycling between plasma membrane and early endosomes (Fig. 3). Overall, more detailed structure-function and electron microscopy studies should be performed to characterize the role of NEDD4-2 and ubiquitination in the intracellular sorting of transporters. However, a striking similarity in the regulation of these processes among various receptor and transporter proteins is already quite evident (Miranda and Sorkin 2007).

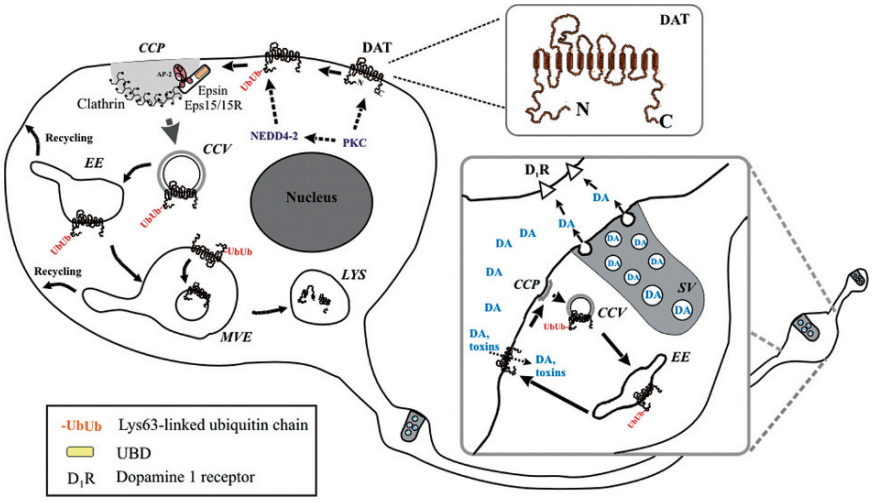

Fig. 3 Hypothetic model of endocytosis and endosomal sorting of DAT. In the somatodendritic part of DA neurons, the activation of PKC results in the NEDD4-2-mediated ubiquitination of DAT. PKC activation can facilitate the NEDD4-2-mediated ubiquitination of DAT either by phosphorylating DAT or DAT-interacting proteins or by activating NEDD4-2. Ubiquitinated DAT is recruited into clathrin-coated pits (*CCP*) by means of interaction with the UBD-containing proteins, such as Eps15/Eps15R and epsin, bound to AP-2 and clathrin in coated pits. After internalization via coated vesicles (*CCV*), DAT is sorted in early endosomes (*EE*) and MVB to lysosomes (*Lys*), presumably by a mechanism similar to that of the EGFR (Fig. 2). In the synapses of the distal axonal processes, DAT is internalized and recycled in a manner similar to that in the neuronal soma, although there is likely no sorting to late endosomes in axonal varicosities because distal axons of dopaminergic neurons lack these late endosomal compartments

6 Conclusions and Outstanding Issues

Ubiquitination has recently emerged as a critical post-translation modification that controls subcellular localization and turnover of transmembrane proteins, many of which are implicated in human neurodegenerative disease and may represent important therapeutic targets. The general consensus is that ubiquitination of the integral membrane proteins mediates the post-endocytic sorting of these proteins to lysosomes. In contrast, the role of ubiquitination in the internalization step of trafficking has been directly demonstrated only for a few endocytic cargoes in mammalian cells. The view that the regulatory functions of ubiquitination in endocytic trafficking are mediated exclusively by mono-ubiquitination has now been questioned. It is now clear that Lys63-linked polyubiquitination is the common modification of many types of transmembrane proteins. It can be proposed that, whereas monoubiquitin binds to most UBDs with low affinity, the linear conformation of Lys63 ubiquitin chains allows multivalent interactions of the same UBD-containing proteins with Lys63-polyubiqutinated cargo, thus increasing the avidity of the interaction, as compared to the interaction with mono-ubiquitin. Further investigation is needed to examine the precise role of Lys63-linked chains in endocytic trafficking.

The role of Lys63-linked polyubiquitination in neurodegenerative disease is emerging. Parkin, a protein frequently mutated in Parkinson's patients, is an E3 ubiquitin ligase that mediates formation of Lys63-ubiquitin chains, and it has been suggested that the aberrant regulation Lys63-linked polyubiquitination may result in Parkinson's disease (Doss-Pepe et al. 2005). In light of the possible role of Lys63-chains in the sorting process in the MVB, it would be interesting to investigate the relationship of the Lys63-ubiquitination and autophagy in neurons. On one hand, several studies demonstrated the important role of MVB and ESCRT complexes in autophagy (Filimonenko et al. 2007; Lee et al. 2007). These data indicate that efficient autophagic degradation requires functional MVBs and provide a possible explanation to the observed neurodegenerative phenotype seen in patients with mutations in the CHMP2B protein a part of the ESCRT III complex. On the other hand, Lys63-linked ubiquitination was found to selectively facilitate the clearance of inclusions via autophagy (Tan et al. 2008). These data indicate that Lys63-linked ubiquitin chains may represent a common modulator of inclusions biogenesis, as well as a general molecule signal targeting cargo to the autophagic system. Since autophagy has a key role in the prevention of the formation of the inclusion bodies in neurodegenerative disease, it is likely that interactions with the ESCRT complexes mediated by the Lys63-polyubiquitin chains in MVBs may be an important step that can be affected during the development of the disease.

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