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COMMENTARY

The ubiquitin-proteasome system and endocytosis

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SUMMARY

Internalization of membrane proteins has been studied for more than three decades without solving all the underlying mechanisms. Our knowledge of clathrin-mediated endocytosis is certainly sufficient to understand the basic principles. However, more detailed insight is required to recognize why different proteins enter clathrin-coated pits with different rates and affinities. In addition to clathrin coat components, at least two adaptor systems and even more accessory proteins have been described to preselect membrane proteins before they can enter cells. Recent experimental data have identified the ubiquitin-proteasome system as a regulatory system for endocytosis. This system is well-known for its basic regulatory function in protein degradation, and controls a magnitude of key events. The ubiquitin-proteasome system is now identified as a regulator of the endocytosis of selected membrane proteins. In this review, we will discuss the complexity and implications of this mechanism for receptor-mediated endocytosis.

Key words: Endocytosis, Downregulation, Ubiquitin, Proteasome, Receptor, Growth hormone

INTRODUCTION

The molecular mechanism of clathrin-mediated endocytosis determines to a great extent the presence of membrane proteins at the cell surface (for review on clathrin-mediated endocytosis, see Mellman, 1996; Schmid, 1997). For many proteins regulation is marginal: they enter the cells via clathrin-coated pits and recycle back from endosomal compartments constitutively. Internalization may be mediated via several amino acid sequences within the cytosolic domain (Mellman, 1996; Trowbridge, 1991). The most common sequences consist of short stretches of amino acids, termed coated pit localization signals. The tyrosine-based motif YXXØ (where X is any amino acid and Ø is an amino acid with a bulky hydrophobic group) is involved in endocytosis of many transmembrane proteins (Marks et al., 1997; Owen and Evans, 1998). Receptors for LDL, transferrin, and asialoglycoproteins are endocytosed via this motif (Chen et al., 1990; Collawn et al., 1990; Spiess, 1990). Internalization of the insulin and β_2-adrenergic receptor is mediated by another well known endocytosis motif (di-leucine) (Haft et al., 1998); in the interleukin-6 receptor, Glut4 and CD4, the di-leucine motif acts in cooperation with an upstream serine (Dittrich et al., 1996; Garippa et al., 1996; Shin et al., 1991). CD3γ and invariant chain are internalized by a di-leucine motif and an upstream aspartic acid (Dietrich et al., 1996; Pond et al., 1995). Other endocytosis motifs have been reported as well. The amino terminus of Glut4 contains a FQQI internalization motif (Garippa et al., 1994; Piper et al., 1993). Most of these proteins recycle back to the cell surface, presumably using the same or similar peptide motifs in the endosomal compartment.

More complex situations exist when plasma membrane proteins enter cells upon stimuli such as hormone binding or specific signal transduction events. Hormone receptors are routed to the lysosomes for degradation after binding to their ligands, and some ion channels, permeases and transporters, like Glut4 and yeast Gap1p, are removed from or translocated to the cell surface depending on the metabolic status of the cell. The mechanisms are diverse and depend on amino acid motifs in the cytosolic tails. Recently, it was shown that the attachment of ubiquitin moieties is involved in internalization of several plasma membrane proteins (reviewed by Hicke, 1997). The ubiquitin conjugation system controls a multitude of regulatory processes via ubiquitin-mediated degradation of essential proteins, and comprises ubiquitin, ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2), ubiquitin ligase (E3) and the 26S proteasome, a multisubunit protease. The enzymes E1, E2 and E3 act in concert and accomplish the covalent attachment of multiple ubiquitin molecules to specific proteins. Polyubiquitinated proteins are degraded by the 26S proteasome (for a recent review on the ubiquitin conjugation system, see Hershko and Ciechanover, 1998). Although it has long been expected that ubiquitin is likely to have roles beyond proteolysis, only recently have the data of Hicke and Riezman (1996), Strous et al. (1996), and

Table 1. Ubiquitinated plasma membrane proteins

Protein	Reference
In yeast	
ABC peptide transporter Ste6p	Kölling and Hollenberg, 1994
Amino acid permease Gap1p	Hein et al., 1995
Multidrug transporter Pdr5	Egner and Kuchler, 1996
α-factor receptor Ste2p	Hicke and Riezman, 1996
a-factor receptor Ste3p	Roth and Davis, 1996
Uracil permease Fur4p	Galan et al., 1996; Marchal et al., 1998
Galactose permease Gal2p	Horak and Wolf, 1997
Maltose transporter	Lucero and Lagunas, 1997
In mammalian cells	
PDGF receptor	Yarden et al., 1986
Prolactin receptor	Cahoreau et al., 1994
GH receptor	Leung et al., 1987
T-cell receptor	Cenciarelli et al., 1992
Fcε receptor I	Paolini and Kinet, 1993
SLF receptor (c-kit)	Miyazawa et al., 1994
EGF receptor	Galcheva-Gargova et al., 1995
FGF receptor	Mori et al., 1995a
CSF-1 receptor	Mori et al., 1995a
Rhodopsin	Obin et al., 1996
p185 (c-erbB-2)	Mimnaugh et al., 1996
Met receptor	Jeffers et al., 1997
Epithelial Na-channel (ENaC)	Staub et al., 1997
Complement receptor 2	Hein et al., 1998

Staub et al. (1997) provide strong evidence that modification of proteins by ubiquitin attachment can have consequences other than direct targeting to the 26S proteasome. In these cases, ubiquitination leads to delayed degradation (probably in the lysosome). Although its function in degradation is indirect, these data indicate a functional role for ubiquitination in the metabolism of plasma membrane proteins.

How ubiquitin is involved in internalization of membrane proteins remains to be shown. Ubiquitin is clearly different from tyrosine- and di-leucine-based endocytosis motifs. These motifs interact with clathrin adaptor AP-2 (Marks et al., 1997; Trowbridge, 1991). Association of AP-2 with plasma membrane proteins results in clathrin binding and coated pit localization. For the β-adrenergic receptor, arrestin serves as an adaptor to induce endocytosis via clathrin coated pits (Goodman et al., 1996). Internalization of CD4 is induced by the adaptor protein Nef which connects the CD4 tail to the AP2/clathrin-mediated endocytic machinery (Foti et al., 1997). Ubiquitin might act as an adaptor between lysine residues in membrane proteins and clathrin (analogous to AP2 between tyrosine-based motifs and clathrin), or between amino acid sequences in the ubiquitin molecule itself and AP2 molecules.

UBIQUITINATED MEMBRANE PROTEINS

Many proteins located in the plasma membrane have been shown to be ubiquitinated (Table 1). In 1986 the first plasma membrane proteins were found to be ubiquitinated when microsequencing of those proteins revealed two amino-terminal sequences. One of them corresponded to the sequence of ubiquitin (Siegelman et al., 1986; Yarden et al., 1986). Since then, numerous cell surface proteins were found to be conjugated to ubiquitin. The actual function of conjugation of ubiquitin to these proteins remained obscure until Hicke and Riezman (1996) showed that ubiquitination of the yeast G protein-coupled α-factor receptor Ste2p in fact marked this plasma membrane protein for proteolysis. They showed that Ste2p degradation occurred in the vacuole, the yeast equivalent of the lysosome, while the receptor was not stabilized in proteasome mutant yeast strains, indicating that the proteasome was not involved in its degradation. Ligand-induced Ste2p ubiquitination depends on serine phosphorylation and results in internalization of the receptor-ligand complex and therefore indirectly mediates vacuolar degradation. In addition, inhibition of endocytosis using the mutant yeast strain *end4*Δ, resulted in an accumulation of ubiquitinated Ste2p at the plasma membrane. This had already been shown for the yeast ABC-transporter Ste6p (Kölling and Hollenberg, 1994) and was later also found for the yeast plasma membrane protein a-factor receptor Ste3p (Roth and Davis, 1996). Ste2p ubiquitination and internalization was inhibited when the lysine residue in the SINNDAKSS sequence was mutated (Table 2). Overexpression of a mutant ubiquitin in which all lysines were changed to arginines did not affect Ste2p internalization, demonstrating that the conjugation of a single ubiquitin molecule to Ste2p lysine residues is sufficient to mediate internalization (Hicke, 1997). This also implies that ubiquitination of Ste2p is not involved in proteasomal degradation, since monoubiquitinated proteins are not a target for the proteasome (Chau et al., 1989). Similar results have been obtained for the yeast galactose transporter Gal2p (Horak and Wolf, 1997). Another yeast membrane protein, the Fur4-encoded uracil permease which spans the membrane ten times is also internalized in a ubiquitin-dependent way. As for Ste2p, inhibition of internalization results in a huge accumulation of ubiquitinated permease at the plasma membrane. In addition, Fur4p degradation occurs in the vacuole, not by the proteasome (Galan et al., 1996; Moreau et al., 1997). In addition, permease polyubiquitination is mediated by ubiquitin ligase (E3) Npi1p/Rsp5p and involves two short Fur4p cytosolic

Table 2. Ubiquitination motifs

Protein	Sequence	Effect	Reference
Cyclins A, B	Destruction box	Degradation by proteasome	Klotzbucher et al., 1996
Ste2p	SINNDAKSS	Internalization, degradation in vacuole	Hicke and Riezman, 1996
Ste3p	PEST-like	Internalization, degradation in vacuole	Roth et al., 1998
Ste6p	DAKTI	Internalization	Kölling and Losko, 1997
GHR	DSWVEFIELD	Internalization, degradation	Govers et al., 1999
Uracil permease	PEST-like	Internalization, degradation in vacuole	Galan et al., 1994
Fur4p	Destruction box	Internalization, degradation in vacuole	Galan et al., 1994
	PEST-like		Marchal et al., 1998
ENaC α/β/γ	PPXYmotif	Internalization, degradation	Staub et al., 1996
IκBα	DSGLDS	Degradation by proteasome	Baeuerle and Baltimore, 1996
β-catenin	DSGIHS (DSGΨXS)	Degradation by proteasome	Aberle et al., 1997
Various proteins	N-term amino acids	Degradation by proteasome	Hershko and Ciechanover, 1998

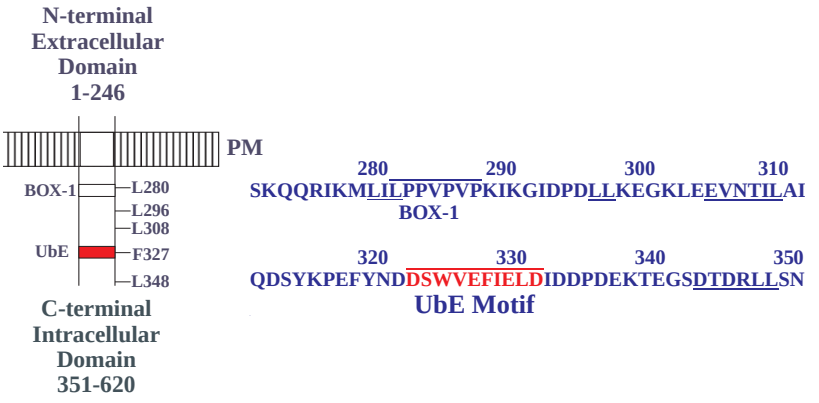

Fig. 1. Structure of part of the cytosolic tail of the growth hormone receptor. Overlined, Box-1 and UbE motif; underlined are possible di-leucine motifs involved in clathrin-mediated endocytosis.

sequences, one identical to the cyclin 'destruction box' RXXLXXXX(N) (Galan et al., 1994; Klotzbucher et al., 1996) and a PEST-like sequence (Table 2) (Marchal et al., 1998). Formation of the ubiquitin chains on the permease takes place via ubiquitin-Lys63, not Lys48, commonly used for ubiquitin attachment (Galan and Haguenauer-Tsapis, 1997). Furthermore, it has been demonstrated that Fur4p internalization is dependent on the presence of the isopeptidase Doa4p, as is the case for the maltose transporter (Lucero and Lagunas, 1997), and that monoubiquitination of Fur4p is already sufficient for internalization. Since endocytosis of the uracil permease requires the 'destruction box', it seems likely that ubiquitination motifs involved in the degradation of certain proteins are also involved in endocytosis of other proteins.

In contrast to the emerging role of the ubiquitin system in endocytosis in yeast, its role in the metabolism of mammalian plasma membrane proteins is less clear. For the PDGF receptor (Mori et al., 1995a,b), the Met receptor (Jeffers et al., 1997), rhodopsin (Obin et al., 1996), and the $p185^{c\text{-}erbB\text{-}2}$ proto-oncogene (Mimnaugh et al., 1996), studies with proteasome inhibitors have indicated that ubiquitination might initiate proteasome action. These findings are supported by data demonstrating the presence of ubiquitinated protein conjugates in the endosomal/lysosomal compartments (Doherty et al., 1989; Laszlo et al., 1990). Together, the studies suggest that the proteasome is involved in (partial) degradation of the cytosolic domains, while the luminal and transmembrane parts are degraded in the lysosome. The role of ubiquitination in endocytosis or degradation of the mammalian T cell antigen receptor (Cenciarelli et al., 1992) and the receptors for IgE (Paolini and Kinet, 1993), SLF (Miyazawa et al., 1994), FGF, CSF-1 (Mori et al., 1995a) and EGF (Galcheva-Gargova et al., 1995) remains unclear. For two mammalian membrane proteins ubiquitination is clearly involved in their internalization: the epithelial sodium channel (ENaC) and the growth hormone receptor (GHR). Internalization of ENaC depends on ubiquitination of its α- and γ-subunits presumably mediated by ubiquitin ligase (E3) Nedd4, the mammalian homologue of yeast Rsp5p. The ligase binds via its WW domains to the PPXY motif in ENaC (Staub et al., 1997; Schild et al., 1996). In patients affected with Liddle's syndrome, a hereditary form of systemic renal hypertension, these PY

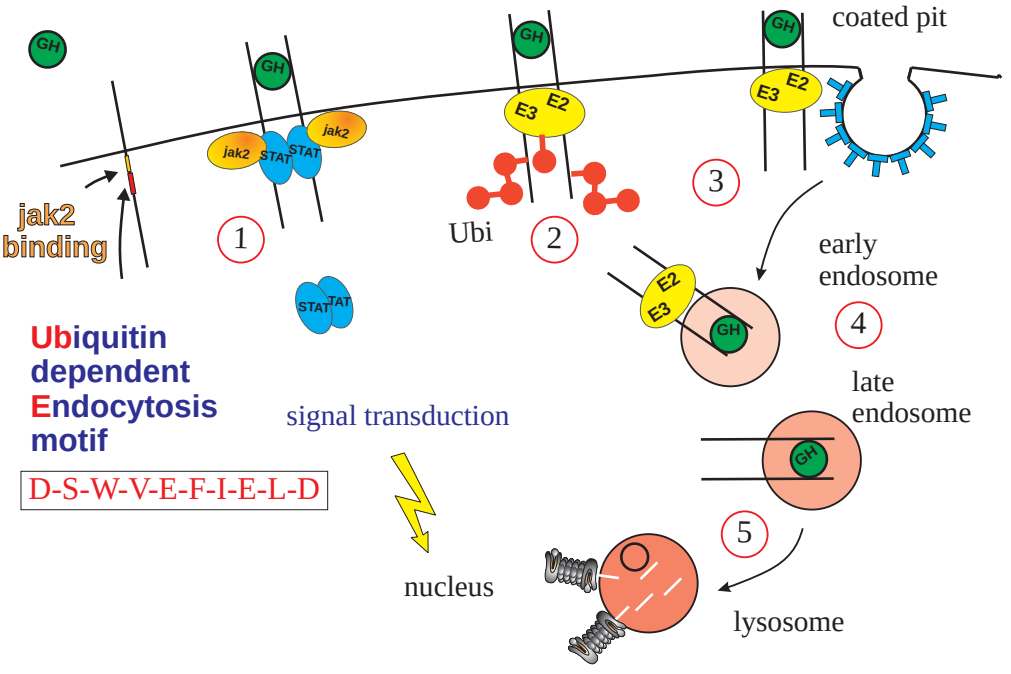

Fig. 2. A scenario for GHR endocytosis and downregulation: (1) GH binds and dimerizes two GHRs, which causes recruitment and activation of Jak2; Jak2 phosphorylates the GHR, itself, and STATs. Signal transduction is relayed to the nucleus and genes are activated; (2) specific ubiquitin conjugases (E2) and ligases (E3) dock onto the UbE motif and ubiquitinate the GHR and possibly other attached proteins; (3) the E2/E3s activate the clathrin-coated endocytosis machinery, serving either as adaptors for clathrin or as connectors to AP2 adaptors; (4) transport to early and late endosomes; (5) degradation of GH and GHR by proteasomes and lysosomes.

Table 3. Proteins containing a potential UbE motif

Sequence	Amino acid residues to PM	Protein
DSWVEFIELD	52	GHR sequence (human, rabbit.....)
DLLVEYLEVD	49	Prolactin receptor, human
DLKGEFIWVD	186	IgE receptor (FcεRII, CD23)
PDGHEYIYVD	17	PDGF receptor α-chain
SDGHEYIYVD	20	PDGF receptor β-chain
EEQCEYLSYD	28	VEGF receptors 2,3
TSEGEYIPLD	24	G protein-act. K^+-channel-1 (IRK1)
STELEYLGPD	30	Insulin-regulated glucose transporter (Glut4)
MDNVDYLTRD		Ca^{2+}-channel, L-type (CIC1)
PVTLDFLDAE		Ca^{2+}-channel, Ltype (CIC2)

motifs are mutated, which leads to an increase of activated channels at the plasma membrane and an increase of Na^+ flux (Schild et al., 1996; Shimkets et al., 1997). The rapid ENaC turnover is affected by inhibitors of both the proteasome and the lysosome (Staub et al., 1997). Ligand-dependent GHR internalization also depends on an intact ubiquitin system (Strous et al., 1996; Govers et al., 1997). Thus, certain mammalian plasma membrane proteins might be degraded by both the proteasome and the lysosome, both pathways being initiated at the plasma membrane by the ubiquitin system.

UBIQUITIN SYSTEM-DEPENDENT ENDOCYTOSIS OF THE GHR

The growth hormone receptor is a member of the cytokine/hematopoietin receptor superfamily, defined on the basis of limited amino acid homology. The extracellular domain contains three pairs of positionally conserved cysteine residues and a WSXWS motif, involved in ligand binding. In the intracellular domain, two motifs have been recognized in several members of this superfamily. A proline-rich motif, referred to as box-1, is situated close to the membrane. It is eight amino acid residues long with a consensus sequence ΨXXXaPXP (Ψ, hydrophobic; X, any amino acid, a, aliphatic; P, proline). The second cytoplasmic motif (box-2) is less conserved and begins with a cluster of hydrophobic amino acid residues and ends with one or two positively charged amino acids. Both domains are involved in signal transduction (Murakami et al., 1991; Tanner et al., 1995). Cytokine receptor family members lack intrinsic kinase activity. Instead, these receptors interact with cytosolic tyrosine kinases of the JAK family upon ligand binding: GHR recruits JAK2 (Wilks and Harpur, 1994). The GHR was initially found ubiquitinated upon amino acid sequencing of the receptor from rabbit liver (Leung et al., 1987). Binding of GH stimulates ubiquitination, internalization, and degradation of the receptor. Moreover, the ubiquitin conjugation system is involved in GHR internalization (Strous et al., 1996; Govers et al., 1997), while at least part of GHR degradation occurs in the lysosome (Murphy and Lazarus, 1984; Yamada et al., 1987). In a Chinese hamster cell line carrying a temperature-sensitive E1 enzyme (ts20), inactivation of E1 results in an accumulation of non-ubiquitinated GHRs at the plasma membrane, while internalization of the transferrin receptor is unaffected (Strous et al., 1996; Govers et al., 1997). These data suggest that GHR ubiquitination and internalization are indeed related.

The 350 amino acid-long GHR cytosolic tail has been examined for its role in endocytosis. If the tail was truncated at amino acid 330 (leaving a 60 amino acid-long tail segment intact) no ligand-induced uptake was observed, despite the presence of three potential di-leucine internalization sequences upstream (Fig. 1) (Govers et al., 1998). Ubiquitin conjugating activity and endocytosis required a specific 10 amino-acid sequence located immediately upstream of D334, which was assessed DSWVEFIELD. In particular, the aromatic as well as the acidic residues did not allow mutation. The sequence was shown to be a ubiquitin-dependent endocytosis (UbE) motif, which does not resemble any other known ubiquitination domain (Table 2) (Govers et al., 1999). Database searches have identified a number of proteins that contain a stretch of amino acid residues, homologous to the GHR UbE motif including the prolactin receptor, which contains high overall sequence similarity to the GHR and has been reported ubiquitinated (Cahoreau et al., 1994). Several other plasma membrane proteins which are known to be ubiquitinated and contain a potential UbE motif include c-erbB-2 (Mimnaugh et al., 1996) and the PDGF receptor (β subunit) (Mori et al., 1992) (Table 3). For the PDGF receptor it has been demonstrated that Tyr579, an essential amino acid residue in the putative UbE motif corresponding to Phe327 in the GHR, is involved in PDGF internalization (Mori et al., 1994). Other potential candidate membrane proteins are the vascular endothelial growth factor (VEGF) receptors 2 and 3, the insulin-responsive glucose transporter Glut4 and several chloride, potassium and calcium channels. A ubiquitinated cytosolic protein which contains a putative UbE motif is protein kinase C. A similar domain is found in subunits of cAMP-dependent kinase and the serine/threonine kinase PCTAIRE.

The experimental data so far indicate that the ubiquitin conjugation system is involved in endocytosis of certain membrane proteins, while most of these membrane proteins are degraded in lysosomes. However, in general the ubiquitin conjugation system cooperates with proteasomes. In all cases discussed above no direct role is allocated to proteasomes. For the Met tyrosine kinase receptor and the PDGF receptor it has been reported that proteasome inhibitors block their degradation. The most likely explanation is that the ubiquitin-proteasome system is involved in transport to the lysosomes in general. This is inferred from studies on lysosome formation from autophagosomes (Lenk et al., 1992). Involvement of proteasomes in the degradation of cytosolic tails has not been established for any of the described proteins. Degradation products have not been detected in the absence or the presence of proteasome inhibitors, not even when antibodies were directed against the extracellular 'proteasome-undegradable' part of these cell surface proteins. This does not exclude proteasome action, as the proteasome might degrade the cytosolic domains of these proteins gradually, during their transport from the plasma membrane to the lysosome. This would result in degradation intermediates of different lengths, which are not easily detectable by immunoblotting. If the GHR is truncated at position 349, a latent di-leucine endocytosis motif (the DTDRLL sequence at position 343) becomes active and the UbE motif is no longer required for ligand-induced internalization (Govers et al., 1998). In addition, the GH uptake rate of truncations longer that 349 is much higher than if this di-leucine motif is removed (unpublished results). The

presence of this internalization signal, which becomes active if a major portion of the tail is removed, suggests that the GHR tail is cut from the C-terminus. Another indication for a direct role of proteasomes in GHR endocytosis comes from experiments with proteasome inhibitors, which show that proteasome inhibitors block both internalization and degradation of the GHR receptor (P. van Kerkhof and G. J. Strous, unpublished results).

The most curious observation is that ubiquitination of the GHR itself is not required for ubiquitin-dependent uptake (Govers et al., 1999). This suggests that GHR internalization requires the recruitment of the ubiquitin conjugation system to the GHR UbE motif, rather than the conjugation of ubiquitin to the receptor. For several plasma membrane proteins it has been suggested that ubiquitination of the protein itself is required for endocytosis (Staub et al., 1997; Terrell et al., 1998). Analogous to the recruitment of JAK2 to the GHR box1 motif, the UbE motif may serve, directly or via adaptor proteins, as an anchoring site for the ubiquitinating enzymes, leading to coated pit localization and subsequently to internalization of the GHR. The most likely scenario for GHR internalization is shown in Fig. 2: binding of the ubiquitin conjugation system to the GHR UbE motif results in the (direct or indirect) interaction of an endocytic adaptor, e.g. AP2. Alternatively, the E2/E3 itself might serve as an adaptor, analogous to the role of arrestin for the β-adrenergic receptor (Goodman et al., 1996). In our model, ubiquitin conjugation to the GHR has no direct role in receptor internalization. Incubation of the ts20 cells at the non-permissive temperature would not only result in inhibition of ubiquitin activation and protein ubiquitination but also in inhibition of the binding of E3 to the GHR. Since certain E3s form a ubiquitin thiolester linkage, binding of these E3s to their specific substrates likely depends on the presence of E1-activated ubiquitin.

If ubiquitination of a GHR-associated protein is involved in receptor internalization, this regulatory protein may well have a more general role in receptor internalization. One potential candidate for this role is Eps15, which is associated with receptors (Fazioli et al., 1993), involved in endocytosis (Carbone et al., 1997; Van Delft et al., 1997a) and ubiquitinated upon receptor activation (van Delft et al., 1997b). Another candidate is Cbl, a well characterized regulator of tyrosine kinase receptors (Miyake et al., 1998); Cbl is ubiquitinated in cells which are stimulated with colony stimulating factor (Wang et al., 1996).

Recently, an anomalous $GABA_A$ neurotransmission due to either a mutation in the gene encoding the E3 UBE3A or to diminished GABRB3 expression was found in a neurological dysfunction called Angelman syndrome (DeLorey et al., 1998; Nicholls, 1998). These observations suggest a connection between the GABA receptor function and the ubiquitin conjugation system, similar as might be expected for GHR regulation if either the UbE motif or the interacting ubiquitinating enzymes were mutated.

CONCLUSIONS

The ubiquitin-proteasome system is recognized as a key regulatory system in basic biological processes like cell cycle regulation, cell growth and proliferation, the rapid degradation of transcriptional regulators involved in a diversity of signal transducing processes and response to environmental conditions. With the recognition that the system also regulates the time span at the cell surface for proteins like hormone receptors and transporters it is especially important to identify the E2/E3 enzymes acting as regulators of these systems. Although it is still too early to discern the common features in the yeast and mammalian systems, it is already clear that the ubiquitin system adds a layer of complexity to the membrane sorting machinery at the plasma membrane. The system might regulate the removal of certain proteins by causing their degradation either in the lysosomes or by proteasomes. A variant would be that the system is merely involved in translocation from the plasma membrane to intracellular compartments. From the list of potential candidates for such a 'special treatment', it is suggestive that in particular proteins involved in anabolic pathways are subjected to this (degradative) system specialized in catabolic pathways.

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