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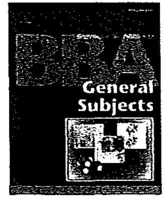

Review

Lectin-like ERAD players in ER and cytosol

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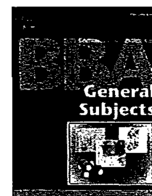

Review

Lectin-like ERAD players in ER and cytosol

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ABSTRACT

Protein quality control in the endoplasmic reticulum (ER) is an elaborate process conserved from yeast to mammals, ensuring that only newly synthesized proteins with correct conformations in the ER are sorted further into the secretory pathway. It is well known that high-mannose type *N*-glycans are involved in protein-folding events. In the quality control process, proteins that fail to achieve proper folding or proper assembly are degraded in a process known as ER-associated degradation (ERAD). The ERAD pathway comprises multiple steps including substrate recognition and targeting to the retro-translocation machinery, retrotranslocation from the ER into the cytosol, and proteasomal degradation through ubiquitination. Recent studies have documented the important roles of sugar-recognition (lectin-type) molecules for trimmed high-mannose type *N*-glycans and glycosidases in the ERAD pathways in both ER and cytosol. In this review, we discuss a fundamental system that monitors glycoprotein folding in the ER and the unique roles of the sugar-recognizing ubiquitin ligase and peptide:*N*-glycanase (PNGase) in the cytosolic ERAD pathway.

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1. Introduction

Approximately one-third of all proteins in eukaryotic cells are targeted to the secretory pathway, and the first compartment encountered by these proteins is the endoplasmic reticulum (ER) [1–3]. The nascent proteins enter the ER in unfolded states through the Sec61 translocon complex [4] and are folded or assembled in the ER through a process that involves various molecular chaperones and folding enzymes. In yeast, the Ssh1p complex has also been identified as a translocon channel in the ER [5,6]. Simultaneously, co- and post-translational protein modifications, such as glycosylation, disulfide-bond formation, and glycosylphosphatidylinositol (GPI)-anchor formation, occur in the ER. Although the ER contains high concentrations of molecular chaperones and folding-assisted enzymes, all newly synthesized proteins do not mature correctly. The ER provides a protein quality control system that monitors protein-folding state and consequently only correctly folded proteins are delivered to downstream compartments (Golgi, endo-lysosomes, and plasma membrane) or secretion [7].

Most proteins in the secretory pathway are glycosylated. Whereas highly diverse oligosaccharides attached to mature proteins are implicated in many extracellular processes, there is evidence that partially trimmed intermediates of high-mannose type *N*-glycans are involved in promoting protein folding, quality control, trafficking, and sorting of glycoprotein [8–10].

The oligosaccharyl transferase catalyzes the transfer of triglucosyl high-mannose oligosaccharide, $Glc_3Man_9GlcNAc_2$, from dolichol pyrophosphate to the asparagine residue in Asn-X-Ser/Thr motifs on the nascent polypeptide chains entering the ER lumen (Fig. 1A). After coupling to the polypeptide, the *N*-linked glycan is trimmed by glucosidase I, glucosidase II, and ER mannosidase I. Monoglucosylated high-mannose oligosaccharide, $Glc_1Man_9GlcNAc_2$, is associated with lectin-type molecular chaperones calnexin or calreticulin (Fig. 1B) [8–10]. The ER houses the deglucosylation–reglucosylation cycle, which consists of glucosydase II and UDP-glucose/glycoprotein glucosyltransferase (UGGT) [11]. These functionally opposite ER enzymes mediate the on/off-cycle in the calnexin–calreticulin system. The repeated cycles of deglucosylation–reglucosylation might be prolonged until the glycoprotein reaches its native conformation [9,12,13]. It is postulated that glycoproteins are allowed to move to the Golgi complex once they achieve correct folding during a certain period of mannose trimming in the ER. Correctly folded glycoproteins are transported from the ER to the Golgi complex through loading onto the transport vesicles. The 53-kDa membrane protein of the ER-Golgi intermediate compartment (ERGIC-53), vesicular integral protein of 36 kDa (VIP36), and VIP36-like (VIPL) have been identified as putative transmembrane cargo receptors, which constitute the family of leguminous type (L-type) lectins [14–17]. These cargo receptors share structural similarities in their lectin domains but exhibit distinct sugar-binding specificities and affinities, which can be finely tuned by sensing pH of each organella [18]. After the correctly folded or assembled glycoproteins move to the Golgi complex, the *N*-glycans receive further trimming of mannose residues and addition of terminal glycosylation to produce diverse *N*-glycan structures [19].

In the ER quality control system, improperly folded proteins or incompletely assembled oligomers are retained in the ER, and are finally retrotranslocated into the cytosol, upon persistence of misfolding,

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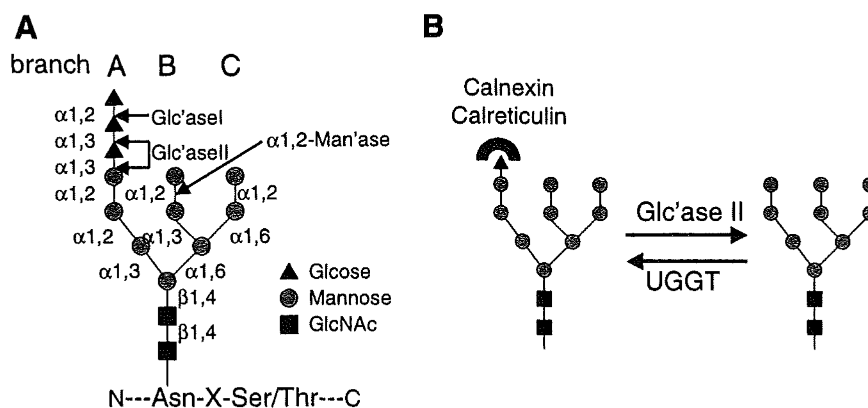

Fig. 1. (A) Composition and structure of $Glc_3Man_9GlcNAc_2$. Linkages are shown and the three *N*-glycan branches are labeled A, B and C. The sites of action of various glycosidases are shown. (B) Lectin-type molecular chaperones calnexin or calreticulin associate with the glucose residue on $Glc_1Man_9GlcNAc_2$. The ER provides a complete calnexin-calreticulin system with deglucosylation–reglucosylation enzymes. Repeated rounds of reglucosylation lead to prolonged association of lectins with the misfolded protein until the glycoprotein reaches its native conformation. Glc'aseI (glucosidase I), Glc'aseII (glucosidase II), α1, 2-Man'ase (α1, 2-mannosidase) and UGGT (UDP-glucose/glycoprotein glucosyltransferase).

where they are degraded by the ubiquitin–proteasome system. This disposal process of folding-defective proteins is called ER-associated degradation (ERAD) [20,21]. It remains elusive how ERAD substrates are selected from proteins that are or might be properly folded. However, recent progress has established that a specific trimming status of *N*-glycan is a signal for the recognition of ERAD substrates. The targeting process as well as the ERAD substrate recognition are mediated by several lectin-type molecules and mannosidases [10,22–24]. It has been shown that the retrotranslocated glycoproteins are ubiquitinated and then deglycosylated by peptide:*N*-glycanase (PNGase) before they are subjected to proteasomal degradation in the cytosol, indicating that *N*-glycosylated proteins are present in the cytosol [25,26]. Indeed, glycoprotein-specific F-box proteins, which are substrate recognition components in the SCF (Skp1-Cullin1-F-box protein) complex type E3 ubiquitin ligase, are found in the cytosol of mammals [27]. This review presents various lectin-type molecules and glycan-modifying enzymes involved in the individual steps of ERAD, which are substrates for recognition, targeting, retrotranslocation, ubiquitination, and degradation by the proteasome (Fig. 2).

2. Substrate recognition and targeting in the ER

2.1. Trimming status of mannose residues on N-glycan as a signal for substrate recognition in ERAD

When the improperly folded or incompletely assembled proteins fail to restore their functional states, they are degraded through the ERAD pathway. The structures of high-mannose type *N*-glycans seem to be important for ERAD in both mammal and yeast. In all eukaryotes, the common structure core glycan, $Glc_3Man_9GlcNAc_2$, is first attached to nascent proteins, then three glucose residues on the A branch are removed by glucosidase I and II (Fig. 1A). Truncation of terminal α1, 2-linked mannose residue on the B branch of high-mannose oligosaccharide is carried out by the ER α1, 2-mannosidase Mns1p in yeast, or the ER α-mannosidase I in mammal. In mammals, the ER α-mannosidase can occasionally remove additional α1, 2-linked mannose residues and generate $Man_{5-7}GlcNAc_2$ as well as $Man_8GlcNAc_2$ structures [28–31]. The finding that this mannose trimming reaction by the ER α-mannosidase is slower than glucosidase I and II reaction led to the concept of the mannose-timer hypothesis [32,33]. Selective inhibition of ER α-mannosidase I with kifnensin and 1-deoxymannojirimycin or Mns1p gene disruption stabilizes misfolded glycoproteins [34–36]. Moreover, overexpression of ER α-mannosidase I accelerates glycan trimming and degradation of misfolded glycoprotein substrates [30]. These observations indicate that the ER α-mannosidase product $Man_8GlcNAc_2$ seems to serve as a degradation signal for ERAD and suggest the presence of lection-like molecule that recognizes $Man_8GlcNAc_2$ or further trimmed structures.

Two types of lectin-like molecules involved in selection and targeting of aberrant proteins for the ERAD pathway were discovered recently. The first is the α-mannosidase-like lectin, EDEM1 and Htm1p/Mnl1p, in mammal and yeast, respectively [37–39]. Although yeast has no paralogue of Htm1p/Mnl1p, two novel EDEM1 paralogues, EDEM2 and EDEM3, have been reported in mammal [40–42]. The other type of lectin-like molecules is a protein that contains mannose 6-phosphate receptor homology (MRH) domain, Yos9p in yeast [43–46], and OS-9 and XTP3-B/ERlectin in mammal [47,48]. These two types of lectin-like molecules are required for degradation of misfolded glycoproteins, but the nature of the glycan degradation signal(s) that is (are) recognized by these lectin-like molecules remains unclear. Recent studies concluded that Htm1p generates a *N*-glycan signal for glycoprotein degradation, which is recognized by Yos9p as described below [49,50].

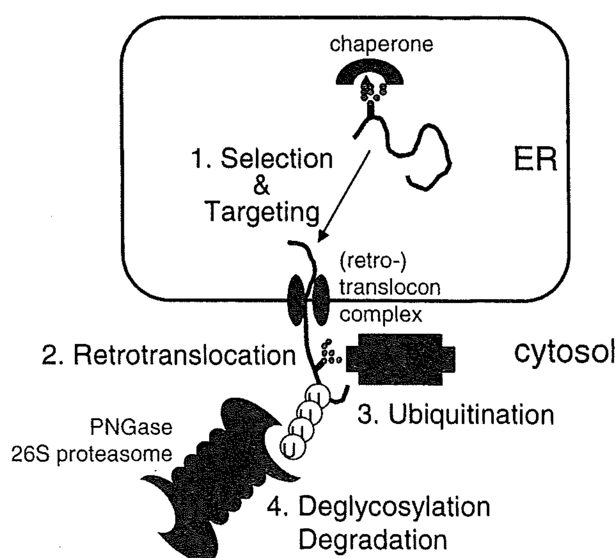

Fig. 2. The ERAD pathway. In step 1, misfolded glycoproteins are recognized by chaperone molecules or enzymes and targeted to the (retro-)translocon complex. In step 2, polypeptides are retrotranslocated from the ER into cytosol through the channel. In step 3, polyubiquitination occurs when the polypeptide chain becomes accessible in the cytosol. In step 4, polypeptides modified by the polyubiquitin chain are degraded by the proteasome. De-ubiquitinating enzymes remove the polyubiquitin chain and deglycosylation by PNGase might also be required for efficient degradation.

2.2. EDEMs in mammal and Htm1p/Mnl1p in yeast

EDEM1 (for *E*R *d*egradation *e*nhancing α-*m*annosidase-like protein) is a type II ER membrane integral protein that has all the signature motifs of class I α1, 2-mannosidases (glycosylhydrolase family 47) but no detectable enzyme activity as a processing α-mannosidase *in vivo* or *in vitro* [38]. In some cell types, EDEM1 is expressed as a soluble luminal protein after efficient cleavage of the signal sequence [38,41]. The gene expression of EDEM1 is up-regulated by ER stress, and overexpression of EDEM1 accelerates the turnover of the null Hong Kong variant of α1-antitrypsin (NHK), an ERAD substrate glycoprotein [38,51]. It has been reported that EDEM1 and calnexin interact with each other and thereby facilitate the transfer of misfolded glycoproteins from the calnexin–calreticulin cycle to the ERAD pathway [51,52]. In yeast, disruption of EDEM orthologue Htm1p/Mnl1p gene delays the degradation of aberrant glycoproteins but not non-glycosylated ERAD substrates [37,39]. Furthermore, EDEM1 associates with derlin-2 and derlin-3, which are retrotranslocation channel candidates [53]. On the other hand, Htm1p/Mnl1p has been implicated in the turnover of ERAD-L pathway of which ERAD substrates have lesions in luminal domain [54]. These observations suggest that EDEM1 and Htm1p/Mnl1p are probably lectins involved in the selection or targeting of misfolded glycoproteins for ERAD, but no direct evidence has been provided yet for interaction between high-mannose type glycans and these lectins. However, EDEM1 has chaperone-like activity that accelerates ERAD by preventing the formation of disulfide-bonded dimers [55] or covalent aggregation [56] containing misfolded glycoproteins.

In mammals, two paralogues, EDEM2 and EDEM3, are identified as soluble ER glycoproteins that accelerate the degradation of misfolded glycoproteins [40–42]. ER mannosidase I, EDEMs and Htm1p/Mnl1p have significant sequence identity in the mannosidase homology region but they differ in the remaining domains (Fig. 3A). Interestingly, EDEMs are inducible proteins whose intraluminal concentrations are increased upon stress-induced activation of the transcription factor Xbp1, a mediator of unfolded protein response (UPR) [41]. RNA interference directed against EDEM proteins [51,57] or inactivation of the Ire1/Xbp1 pathway of UPR results in sub-optimal disposal of misfolded glycoproteins [58], prevents protein folding and reduces secretory capacity [59].

EDEM3 was recently reported to have mannosidase activity *in vivo*; overexpression of this protein results in mannose trimming to $Man_7GlcNAc_2$ and $Man_6GlcNAc_2$ [42]. Surprisingly, overexpression of EDEM1 can also accelerate the removal of a terminal mannose residue from the A branch [56]. These observations suggest that the EDEM family proteins carry α-mannosidase activities involved in the extraction of misfolded glycoproteins from the calnexin–calreticulin cycle rather than acting as lectin [56]. Furthermore, in yeast, overexpression of Htm1p/Mnl1p also produces $Man_7GlcNAc_2$ glycan. By using various mutant strains, Clerc et al. [50] defined the function of Htm1p/Mnl1p as a α1, 2-specific exomannosidase, which generates the $Man_7GlcNAc_2$ glycan with a terminal α1, 6-linked mannose residue. This terminal α1, 6-linked mannose residue produced by Htm1p/Mnl1p is recognized by Yos9p as the glycan signal for destruction of glycoproteins through the ERAD pathway [49].

2.3. OS-9 and XTP3-B in mammal and Yos9p in yeast

Yos9p (yeast osteosarcoma 9) was discovered in a genetic screening to identify components that participate in glycoprotein turnover [43]. Yos9p is a glycoprotein containing an N-terminal signal sequence, a MRH domain, and a C-terminal HDEL peptide that retains it in the ER lumen (Fig. 3B).

Studies using yeast indicate that proteins are targeted to the appropriate ERAD pathway based on the site of the misfolded lesion [54,60,61]. Substrate proteins with lesions in the cytoplasmic, luminal, and membrane-spanning domains follow ERAD-C, ERAD-L, and ERAD-M pathways, respectively. Two ER-associated E3 ubiquitin ligases, Hrd1p/Der3p complex and Doa10p, are involved in the ERAD pathway in yeast [62–64]. ERAD-L substrates that include membrane and soluble proteins with luminal lesions use Hrd1p/Der3p complex, while ERAD-C substrates use a Doa10p-containing complex. It seems that ERAD-M substrates also use Hrd1p/Der3p complex. It was reported that Yos9p plays a critical role in the recognition and/or targeting to ERAD of soluble and membrane bound proteins by using pathway-specific substrates [44,45]. Moreover, Yos9p is recruited into a protein complex containing the Hrd1p/Der3p and the luminal chaperone Kar2p through association with Hrd3p, a luminally-exposed transmembrane tetratricopeptide repeat (TPR)-containing protein known to associate with Hrd1p [62–64]. Thus, Yos9p is a critical component of ERAD-L pathway [46].

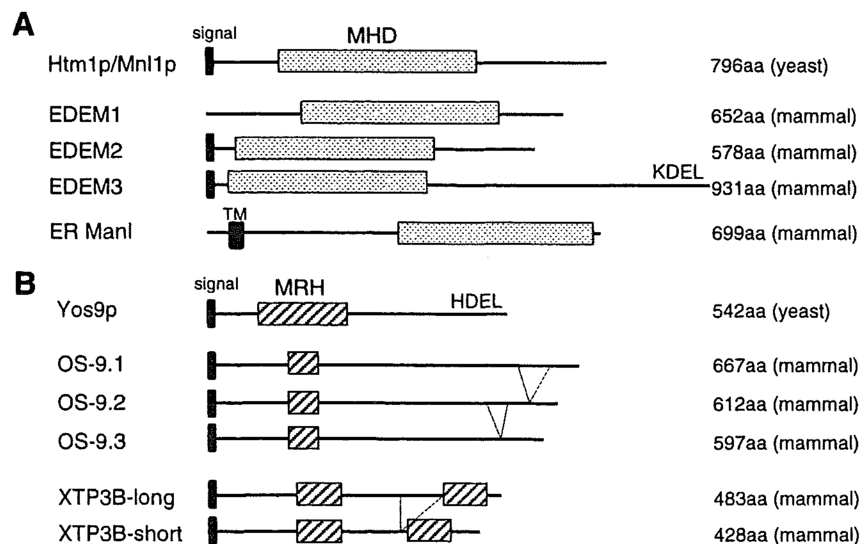

Fig. 3. The domain structure of Htm1p/Mnl1p and Yos9p protein families. (A) Yeast Htm1p/Mnl1p and its mammalian orthologues EDEM family. The mannosidase homology domain (MHD), signal sequence, C-terminal KDEL (ER retention signal), and transmembrane domain (TM) are shown. (B) The mannose 6-phosphate receptor homology (MRH) domain containing proteins. Conserved MRH domains, signal sequence, and C-terminal HDEL (ER retention signal) are shown. Alternative splicing generates variant forms; i.e., the regions depicted by dashed lines are deleted.

Disruption of Yos9p abrogates the degradation of the glycosylated but not the non-glycosylated forms of CPY*, a typical ERAD substrate [43–46]. Furthermore, Yos9p mutations that replace conserved residues in the carbohydrate-binding domain, reduce the rate of misfolded glycoprotein degradation, suggesting that Yos9p acts through the binding of glycans [44,46]. Yos9p can discriminate between mutant CPY* and wild-type CPY, and perhaps to bind glycoproteins carrying $Man_8GlcNAc_2$ and $Man_5GlcNAc_2$ glycans. Recently, Quan et al. [49] characterized the glycan-binding specificities of recombinant Yos9p by using frontal affinity chromatography and found that it recognizes glycans containing terminal α1, 6-linked mannose residues. Other Htm1p/Mnl1p and Yos9p studies showed that Htm1p/Mnl1p acts upstream of Yos9p to mediate the generation of glycans containing terminal α1, 6-linked mannose residues as the glycan signal for ERAD (Fig. 4).

In mammal, two Yos9p orthologues, OS-9 and XTP3-B/Erlection, which contain one and two MRH domains, respectively, were recently characterized as ER-resident luminal glycoproteins [47,48]. OS-9 has two or three transcriptional variants and their depletion causes significant impairment of the glycosylated ERAD substrate NHK degradation [47]. In comparison, knockdown of XTP3-B has no effect on NHK clearance [47], but overexpression of XTP3-B retards the degradation of NHK [48]. XTP3-B also has two transcriptional variants and only the long isoform strongly inhibits glycan-less mutant NHK-QQQ [48]. The mutant form transthyretin, a non-glycosylated serum protein, is also bound to XTP3-B but not OS-9 [47]. Although no interaction between OS-9 and XTP-B is found, OS-9 interacts with GRP94, the ER-resident Hsp90 paralogue. Knockdown of GRP94 stabilizes NHK, indicating that GRP94 is required for degradation of ERAD substrate [47]. Thus, OS-9 and XTP3-B are both glycoproteins containing MRH domain(s) but each has distinct substrate recognition mode and associates.

Both proteins, except for the short form of XTP3-B, associate with Hrd1 ubiquitin ligase complex through SEL1L (suppressor of lin-12-like protein, the mammalian Hrd3p orthologue), a multiply glycosylated type I ER membrane protein. Christianson et al. [47] proposed that the MRH domains of OS-9 and GRP94 are required for interaction with SEL1L but not substrates. The conclusion that mannose binding is used to target quality control sorting receptors to the ER membrane ubiquitin ligase complex rather than for substrate recognition is in conflict with Yos9p studies from yeast. However, very recent study by Hosokawa et al. revealed that OS-9 recognizes *N*-glycans on NHK but not SEL1L and that the lectin activity of OS-9 is required to enhance glycoprotein ERAD [65]. The OS-9 MRH domain specifically binds to *N*-glycans lacking the terminal mannose from the C branch generated by some mannosidase not determined. Further studies are required for a better understanding of the complete mechanism of selection and targeting of ERAD substrates.

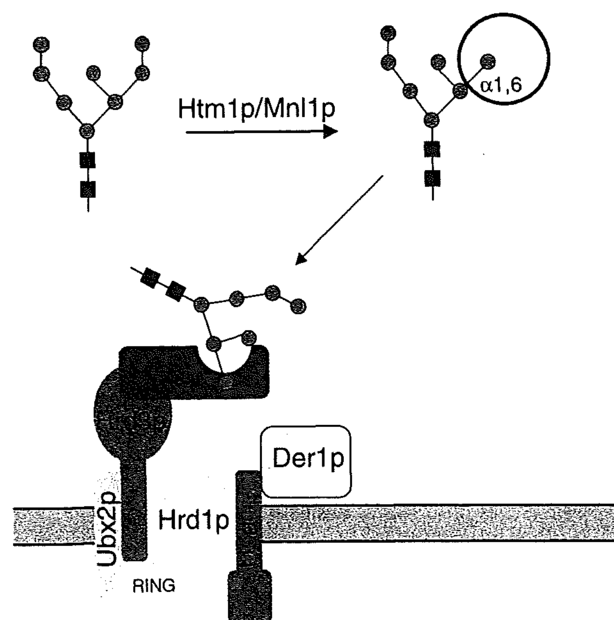

Fig. 4. Model of dual recognition of glycoprotein substrates in yeast ERAD pathway [49]. Htm1p/Mnl1p has α1,2-specific exomannosidase activity for high-mannose type *N*-glycans and produces the terminal α1, 6-linked mannosyl residue (marked with circle), which is recognized by Yos9p in the components of the Hrd1p ubiquitin ligase complex (see text for details).

3. Ubiquitination and proteasome degradation in the cytosol

In the ERAD pathway, protein ubiquitination participates in both protein extraction from the ER into the cytosol and subsequent proteasomal degradation [20]. Protein ubiquitination requires the action of an E1 ubiquitin-activating enzyme, E2 ubiquitin-conjugating enzymes, and E3 ubiquitin ligases. In this ubiquitin pathway, E3 is responsible for the selection of target proteins [66]. In yeast, RING-finger type E3 ubiquitin ligases, Hrd1p and Doa10p, have been implicated in the degradation of every studied ubiquitinated ERAD substrate [54,63]. Mammalian orthologues of Hrd1p and Doa10p have been identified [67,68], but the repertoire of E3s involved in the ERAD pathway is greatly expanded in mammals [21,22]. Since bulky *N*-glycans on proteins can impair their entry into the interior of the cylinder-shaped proteasome, *N*-glycans are postulated to preclude proteasomal degradation of ubiquitinated substrates. PNGase is a cytosolic deglycosylation enzyme and the reaction catalyzed by this enzyme is considered important for subsequent proteasomal degradation [69]. In this section, we describe the glycoprotein-specific E3s and PNGase.

3.1. Fbs proteins

E3s constitute a diverse family of proteins or protein complexes. One of the best-characterized families of E3s is the SCF complex, consisting of common components, Skp1, Cullin1, and Roc1/Rbx1, and a variable component F-box protein (Fig. 5A). The F-box protein consists of an F-box domain that binds to Skp1 and a C-terminal substrate-binding domain. Human and mouse genomes encode 69 and 74 F-box proteins respectively, and a large number of F-box proteins function in the specific ubiquitination of a wide range of substrates [70]. Fbs1 (*F-box* protein that recognizes sugar chain *1*) is a glycoprotein-specific F-box protein, which is identified as a novel member of the ERAD-linked E3 component in mammal [71]. In addition to Fbs1, at least four F-box proteins with high homology to the C-terminal substrate-binding domain have been reported in human [72,73]. These F-box proteins cluster into two groups as demonstrated by phylogenetic analysis (refer to TreeFam in Sanger institute): one group is composed of Fbs1/Fbg1/Fbx2/NFB42, Fbs2/Fbg2/Fbx6b and Fbg3/Fbx44, and the other of Fbg4/Fbx17 and Fbg5/Fbx27 (Fig. 5B). In the human and mouse genomes, these two groups of genes are located separately, but the genes in each group are arranged in tandem with very short intergenic regions [72]. These Fbs family proteins are conserved in mammals and *C. elegans* has two hypothetical F-box proteins (C14B1.3 and T01E8.4), which show significant homology to this family proteins [72].

The expression of Fbs1, Fbg4, and Fbg5 is tissue-specific, but that of Fbs2 and Fbg3 is relatively ubiquitous. Among the Fbs family members, Fbs1 can bind strongly to the high-mannose type glycans, while Fbs2 and Fbg5 bind them more weakly, but no sugar-binding activities have so far been detected for Fbg3 and Fbg4 [27,71,74,75]. To gain further insight into Fbs family proteins, identification of endogenous substrates of Fbg3 and Fbg4 will be essential.

3.1.1. Function of Fbs1

Fbs1 can bind to proteins modified with high-mannose type *N*-glycans in the ER, while the physical association between Fbs1 and its

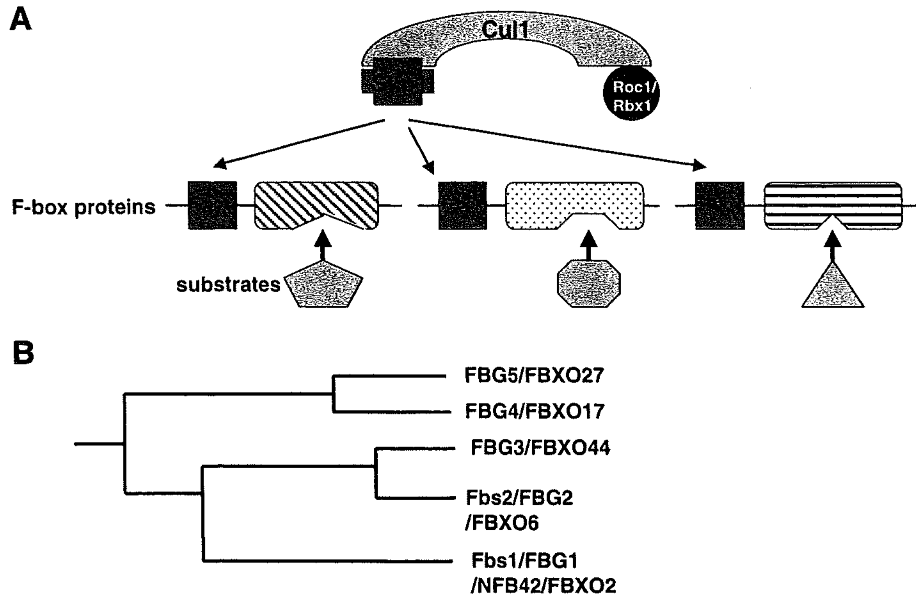

Fig. 5. (A) Structure of SCF complex. In this type E3, cullin1 (Cul1) functions as a molecular scaffold that simultaneously interacts at the amino terminus with the crucial adaptor subunit, Skp1 and at the carboxyl terminus with a RING-finger protein, Roc1/Rbx1, and a specific E2. Skp1 binds to one of many F-box proteins through the F-box domain. (B) Phylogenetic tree of Fbs protein family [73].

substrates, such as pre-integrin β1, is detected in the cytosol in the presence of the proteasome inhibitor. In addition, overexpression of the dominant-negative form of Fbs1 lacking the F-box domain is associated with inhibition of the degradation of ERAD substrates, thus suggesting that SCF^{Fbs1} is involved in the ERAD pathway [71]. Fbs1 is expressed in all major areas of the brain [76]. Indeed, it has been reported that SHP substrate-1 (SHPS-1), a transmembrane glycoprotein abundantly present in the central nervous system [77], and NR1, a subunit of N-methyl-D-aspartate (NMDA)-type glutamate receptors that plays a central role in neuronal development and information storage in the brain, are degraded through the ERAD pathway, and such degradation is mediated by SCF^{Fbs1} [78].

A recent study reported that Fbs1 is essential in inner-ear homeostasis [79]. Orthologues of Fbs1 and Skp1 have been identified in the guinea pig as abundant proteins in the organ of Corti, the sensory organ of the cochlea, where they are called OCP1 and OCP2, respectively [80]. Furthermore, mice with targeted deletion of Fbs1 develop age-related hearing loss with cochlear degeneration and high cochlear levels of the inner-ear gap junction protein, connexin26, which interacts with OCP1 [81], have been described in Fbs1-deficient mice [79].

Although Fbs1 can form the SCF^{Fbs1} complex as an E3 [82], the major part of Fbs1 is present as Fbs1–Skp1 heterodimer or Fbs1 monomer but not in SCF^{Fbs1} [83]. The restricted presence of Fbs1 as SCF^{Fbs1} complex is found in the fraction bound to the ER membrane and the SCF^{Fbs1} complex is associated with p97/VCP, a cytosolic ATPase member of the AAA ATPase family [84]. It has been suggested that the p97–Ufd1–NPl4 complex is required for extraction of misfolded proteins from the ER into the cytosol [85]. Moreover, extraction of the substrate of SCF^{Fbs1} seems to be dependent on the ATPase activity of p97, suggesting that SCF^{Fbs1} is positioned in the ER membrane in such a way that it can ubiquitinate substrates immediately after retrotranslocation to the cytosol [84]. Recent data suggest that the inefficient SCF complex formation of Fbs1 is due to the short linker sequence between its F-box domain and the substrate-binding domain [83]. Fbs1 can prevent the aggregation of glycoproteins *in vitro* and *in vivo*. Since the Fbs1 protein is abundant in neuronal tissues, it is likely that Fbs1 assists in the clearance of aberrant glycoproteins in neuronal cells by suppressing aggregation formation, independent of the E3 activity [83]. Hence, Fbs1 has an additional function as a unique chaperone for these proteins.

3.1.2. *Mechanism of N-glycan recognition by Fbs1*

Fbs1 and Fbs2 interact with glycoproteins containing high-mannose type *N*-glycans. Pull-down analysis using various oligosaccharides revealed that $Man_{3-9}GlcNAc_2$ is required for efficient Fbs1 binding, while modifications of mannose residues by other saccharides and deletion of an inner GlcNAc residue diminished its binding, suggesting that it specifically recognizes the inner chitobiose structure (GlcNAc-GlcNAc) in the *N*-glycan [74]. X-ray crystallographic and nuclear magnetic resonance (NMR) data indicate that the sugar-binding domain of Fbs1 is composed of a 10-stranded β-sandwich with two α-helices, and binds the $Man_3GlcNAc_2$ portion of oligosaccharides through the loops connecting the β-strands (Fig. 6A) [86,87]. In general, the internal chitobiose of *N*-glycans in native glycoproteins is shielded by the amino acid residues surrounding the glycosylation site, and therefore is unlikely to make contact with Fbs1. The finding that Fbs1 and Fbs2 interact more efficiently with denatured glycoproteins than with native ones led to the hypothesis that the innermost position of *N*-glycans becomes exposed upon protein denaturation and is used as a signal by unfolded glycoproteins to be recognized by these E3s (Fig. 6B) [84]. Thermodynamic analysis of interactions between synthetic *N*-glycan and Fbs1 has revealed that $Man_3GlcNAc_2$ has a substantially stronger affinity than high-mannose type glycans $Man_{8-9}GlcNAc_2$, probably because the outer mannose residues cause steric repulsion [88]. This is consistent with trimming status mannose residues on *N*-glycans of ERAD substrates prior to emergence into the cytosol [28–31].

3.2. *PNGase*

PNGase was originally thought to contribute to the ERAD pathway because deglycosylation of glycoproteins should increase the efficiency of proteasomal degradation of the substrate [69]. Indeed, the results of RNA interference (RNAi) and inhibitor studies show the involvement of PNGase in the ERAD pathway [69,89], and degradation of a ricin toxin A-chain mutant mediated by ERAD pathway is dependent on PNG1 [90].

PNGase belongs to the transglutaminase (TGase) superfamily and its catalytic triad consisted of Cys-His-Asp is essential for enzyme activity [91–93]. The gene encoding the cytoplasmic PNGase was first identified in yeast as PNG1, and is conserved throughout eukaryotes [93]. While yeast PNG1 possesses a core catalytic domain, PNG1 orthologues in

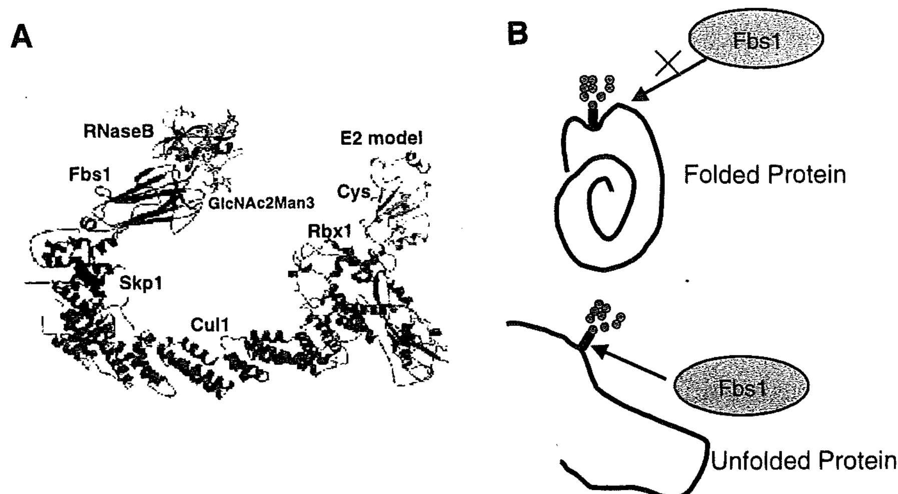

Fig. 6. (A) Structural model of SCF^{Fbs1}-RNaseB-E2 complex constructed based on the crystal structures of Fbs1-Skp1, Skp1-Cul1-Rbx1m, the E2 subunit of the c-Cbl-UbcH7 structure, and the sugar-binding domain of Fbs1-RNaseB structure [81]. (B) A hypothetical model depicting the molecular mechanism of substrate recognition by Fbs1. The intramolecular interaction of the innermost GlcNAc with the polypeptide moiety generally hampers the binding of Fbs1 to chitobiose portions of glycoproteins as a result of steric hindrance in their native states. Fbs1 recognizes the innermost position of N-glycans as a signal for unfolded glycoproteins.

higher eukaryotes possess additional domains flanking the core domain (Fig. 7).

Yeast PNG1 closely associates with the 26S proteasome through interaction with the DNA-repair protein Rad23p [94]. Mammalian PNGase also bind to HR23A/HR23B, Rad23p orthologues [95]. Rad23p or HR23 contains an N-terminal Ubl domain, which interacts with the proteasome, and two UBA domains, which interact with ubiquitin, suggesting its shuttling role of substrate to the proteasome [96]. It is well known that Rad23p also binds to Rad4p (XPC protein) and participates in the DNA-repair events [97]. Interestingly, the Rad4p-binding domain of Rad23p is also responsible for interaction with the core domain of PNGase [98]. Based on the crystallographic data of yeast PNG1–Rad23p complex bound to a PNGase irreversible inhibitor, Z-VAD-fmk, PNG1 comprises three domains, a catalytic domain, a Zn-binding domain, and a Rad23-binding domain. The active site of PNGase is located in a deep cleft, which is sufficiently wide to accommodate unfolded polypeptides but not open for native glycoproteins, thus explaining the specificity of PNGase for denatured substrates [99].

PNG1 orthologues in higher eukaryotes carry additional domains. In mammalian PNGase, the N-terminal PUB (Peptide: N-glycanase/UBA or UBX-containing proteins) domain, a novel protein–protein interaction site [100], and the C-terminal mannose-binding domain, which presumably contributes to the oligosaccharide-binding specificity of PNGase [101] are found in addition to the core domain. The activity of the truncated PNGase mutants lacking the C-terminal domain is significantly reduced, suggesting that the presence of the

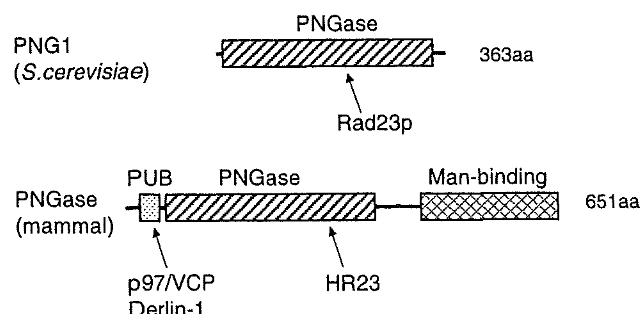

Fig. 7. The domain structure of yeast PNG1 and mammalian PNGase. The interacting proteins for N-terminal PUB domain and catalytic core domain (PNGase) are shown. The PNGase domain comprises three domains, a catalytic domain, a Zn-binding domain, and a Rad23-binding domain.

domain may accelerate substrate turnover. Interestingly, the crystal structure of the C-terminal domain of mouse PNGase reveals that the β-sandwich architecture resembles the sugar-binding domain of Fbs1 and accommodates the Manα1-6(Manα1-3)Manα1-6 moiety of N-glycans in a saddle-shaped depression at one end distal to the N-and C-termini of this domain [101]. Therefore, the C-terminal domain seems to capture the mannosyl branches of high-mannose type N-glycans, leaving their sugar-peptide junction oriented toward the active site, and enhances the catalytic activity.

The N-terminal PUB domain is thought to be involved in the ubiquitin-related pathway based on the engagement of this domain in interactions with Derlin-1 and p97/VCP [102–104]. The ERAD machinery is likely to form an efficient glycoprotein degradation complex for ubiquitination, deglycosylation, and proteasomal degradation, especially in mammals, because PNGase is associated with ERAD substrate extraction machinery (p97/VCP or derlin-1) through the PUB domain and the proteasome through the binding of its core domain with HR23.

It is not clear whether deglycosylation precedes, follows, or occurs simultaneously with ubiquitination. To recognize glycoprotein substrates by SCF^{Fbs1} or SCF^{Fbs2}, it is necessary that these glycoprotein-specific E3s ubiquitinate the substrates prior to deglycosylation by PNGase.

4. Perspectives

The protein quality control in the ER, which exploits N-glycans as tags for incompletely folded proteins, is conserved from yeast to mammals. Since N-glycan in the ER represents a conserved structure in all eukaryotic cells, different from diverse N-glycans expressed on the surface of cells, it is appropriate to provide a common role for N-glycans in protein quality control of many different proteins. The repertoire of N-glycan recognition molecules and enzymes, however, are diverse and complex in higher eukaryotes compared with yeast. In assisting protein folding as the first stage of quality control in the ER, the calnexin/calreticulin cycle is incomplete in *S. cerevisiae* because reglucosylation enzyme UGGT has not been found. Correspondingly, in the second stage of glycoprotein quality control in the ERAD pathway, higher eukaryotes have acquired diversified system.

First, multiple mammalian orthologues of Htm1p/Mnl1p or Yos9p exist, such as EDEM1, 2, and 3, and OS-9 and XTP3-B. In yeast, the refined model of Htm1p/Mnl1p as an α-mannosidase produces terminal α1, 6-linked mannose residues, as a targeting signal for degradation, which is recognized by Yos9p. However, the mechanisms involved in the

recognition of the unfolded status of ERAD substrates by the Htm1p/Mnl1p are not fully understood. All EDEMs, as well as the ER α-mannosidase I, accelerate ERAD of misfolded glycoproteins such as NHK, but *in vivo* differences in their substrate-specificities and enzymatic activities remain poorly understood. Both OS-9 and XTP3-B possess alternative splicing variants and their substrate recognition mechanisms and associates are likely different.

Second, mammalian PNGase possesses N-terminal PUB domain in addition to the core domain, which is responsible for catalytic activity and association with 26S proteasome thorough HR23. The PUB domain contributes to the recruitment of the PNGase-26S proteasome complex to the ER through the ERAD machinery on the ER such as p97 or Derlin-1. On the other hand, yeast PNG1 fails to associate with p97, but not the Rad23p-proteasome, because it lacks the PUB domain. In the conserved ERAD pathway, why are there differences between yeast and mammals?

Third, there are several ERAD-E3 variants in mammals. In addition to Hrd1 and Doa10, gp78, CHIP, Parkin, RNF5/RMA1, and $SCF^{Fbs1,2}$ have been characterized as the ERAD E3s in mammals [21]. As described in this review, Fbs proteins recognize the exposed innermost chitobiose moiety in aberrant glycoproteins as a tag for incompletely folded proteins, but the recognition systems of Fbs proteins and C-terminal domain of PNGase have not been found in yeast. Interestingly, the C-terminal mannose-binding domain of PNGase appears in *C. elegans* PNGase1 [25], and the orthologues of Fbs proteins are also found in *C. elegans* gene although the *N*-glycan-binding activities have not been reported in this species [72]. With regard to Fbs1, the major part of the protein is not present as SCF^{Fbs1} E3 complex, suggesting that Fbs1 plays other roles beside ERAD E3. For example, several *N*-glycosylated proteins are found in the cytosol (for review, see [26]). It is possible that Fbs1 regulates such glycoprotein in the brain as a chaperone molecule.

In the conserved ERAD pathway, how can we explain the presence of so many complex ERAD system in mammals than yeast? And what functions do they serve? Answers to these questions require further studies with the aim to provide a better insight into the molecular mechanisms of mammalian ERAD.

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Chapter 4
Protein Misfolding and Axonal Protection in Neurodegenerative Diseases

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Abstract Genetically engineered mouse model studies show that neuronal dysfunction caused by protein aggregation/misfolding are reversible, indicating that injured neurons are alive even under disease states. Protein misfolding/aggregation in axons and distal dominant axonal degeneration are observed in a subgroup of degenerative diseases and in certain experimental conditions. Moreover, therapeutic approaches towards axonal protection are effective in neurodegenerative disease mouse models; (a) axonal regeneration, (b) anti-Wallerian degeneration, (c) autophagy enhancement, and (d) stabilization of microtubules. These studies demonstrate that axonal protection/functional repair of axons can be general therapeutic interventions for neurodegenerative diseases.

4.1 Neuronal Dysfunction in Neurodegeneration is a Reversible Process

It had been believed that neurodegeneration is not reversible. However, recent studies of transgenic mouse models, which express abnormal proteins associated with Alzheimer's disease, diffuse Lewy body disease, Parkinson's disease (PD), Huntington's disease (HD) and tauopathies such as frontotemporal dementia develop distinct disease-related neurological impairments, elegantly show that some neurological deficits of neurodegenerative cascades can be prevented or reversed by removing abnormal proteins, without obvious alteration of the number of neuronal cell bodies [1]. Thus, neurological impairments that are associated with neurodegenerative conditions might be caused by neuronal dysfunction to some extent rather than neuronal loss. These studies also demonstrate that symptoms arise from neuronal dysfunction which precedes neuronal death [1]. In HD mice model, as in most of the other triplet repeat diseases, the mutant huntingtin proteins form misfolded nuclear aggregates,

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which are highly insoluble. The double mutant huntingtin transgenic mice, in which the bidirectional transgene expression is activated by the removal of doxycycline at birth, express high levels of both mutant huntingtin and lacZ in the striatum, cortex, and hippocampus [2, 3]. Most of striatal neurons are stained with an anti-huntingtin antibody, showing diffuse nuclear aggregates. By 8 weeks of age, striatal morphological alterations in the mutant huntingtin transgenic mice include a reduced size, reactive gliosis, and a decrease in D1 receptors (a feature seen in HD patients). All of the mice at this age also show a behavioral abnormality common to mouse models of HD: when suspended by their tails, they clasp their limbs. This behavioral phenotype was aggravated over time. Neuropathological examination demonstrated the colocalization of various molecular chaperones, ubiquitin, and proteasome subunits with the aggregated proteins. Surprisingly, abolishing the expression of mutant huntingtin by Cre-loxP system in mutant huntingtin transgenic mice with neurodegenerative phenotype results in either a halt of the disease progression or a full recovery from the disease phenotype including pathological changes [2, 3]. This observation indicates that irreversible changes that commit the neurons to persistent dysfunction or death do not necessarily take place in the neurodegenerative process. These observations suggest that therapeutic approaches aiming at elimination of misfolded proteins might be effective in treating neuronal dysfunction. Furthermore, the recovery from motor disturbances indicates that plastic changes can occur when the toxic insult ceases [3]. Recent studies of mutant huntingtin transgenic mice show that the neuronal dysfunction may be caused by misfolded mutant huntingtin protein, at synaptosomal proteasome and mitochondria, which seem to trigger vicious cycles of aberrant neuronal activity [4].

4.2 Neuronal Dysfunction Is Not Treatable by Anti-Cell Death Therapy

Although an important role of apoptosis is implicated in neurodegenerative diseases, data from both humans and animal models indicate that neurodegeneration is often a long-lasting process that finish with cell death only after a prolonged period of disease state.

In PD model mice study, although peptide inhibitors of caspases block 1-methyl-4-phenylpyridinium (MPP+)-induced dopaminergic neuronal death, dopaminergic neuronal terminals are not rescued [5]. Similarly, adenovirus-mediated transgene expression of X-linked inhibitor of apoptosis protein (XIAP) blocks death of dopaminergic neurons in a N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced PD mouse model, but does not prevent the decrease of dopaminergic terminal markers in the striatum [5]. Moreover the resistance of the dopaminergic neurons in the pro-apoptotic Bax protein knockout mice against MPTP toxicity is accompanied by a significant, although less prominent, sparing of striatal dopamine contents [6]. In the superoxide dismutase 1 (*SOD1*) transgenic mouse models of amyotrophic lateral sclerosis (ALS) study, we have also shown that overexpression

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of XIAP in spinal motor neurons rescues cell bodies of motor neuron without inhibition of neuronal dysfunction [7]. Similarly, removal of *Bax* gene resulted in complete rescue of cell bodies of motor neurons in ALS model mice, but denervation and axonal degeneration still occurred [8, 9]. Moreover with Bcl-2 transgenic mice crossed with *pmn* mice to block cellular apoptosis, motor neurons were completely rescued, but motor axons degenerate to the same extent as in *pmn* mice with normal levels of Bcl-2, and there is no change in muscle strength or life span [9, 10]. Consistent with these findings, although an important role for synaptic caspase activation and apoptosis has been proposed, axonal degeneration after withdrawal of trophic support occurs without activation of caspases in contrast to cell death of the cell body [9, 11]. These studies support the idea that axonal degeneration/dysfunction may proceed independently from the molecular events regulating cell death, and that apoptosis plays a critical role in neuronal cell body death, and neuronal dysfunction is not treatable by anti-cell death therapy. Therefore, anti-dysfunction therapies which target axonal degeneration/dysfunction are promising for treatment of neurodegenerative diseases.

4.3 Morphological Aspects of Neuronal Dysfunction Caused by Protein Aggregation/Misfolding in Human Neurodegenerative Disorder

It is hard to morphologically evaluate the neuronal dysfunction caused by protein aggregation/misfolding in the central nervous system, because neurons possess intricate three-dimensional structure, and are embedded deep in the brain which prevents accurate observation of cell shape. In contrast, morphological evaluation of the peripheral nervous system shows that degeneration of the cardiac sympathetic nerve occurs in PD and diffuse Lewy body disease, both of which are caused by accumulation of misfolded α-synuclein, and that degeneration of their distal axons precedes loss of their neuronal cell bodies in the paravertebral sympathetic ganglia [12]. This interesting observation suggests that distal dominant axonal degeneration precedes cell death not only in peripheral sympathetic, but central dopaminergic neurons of PD. Moreover, it is implicated that the centripetal degeneration may represent the common pathological process underlying various neurodegenerative disorders.

In ALS study, there are data supporting the hypothesis that the pathology of ALS starts with distal axonal degeneration [9]. Neuropathological studies, by quantitative morphometry, demonstrate a distal-to-proximal gradient of axonal pathology in phrenic nerves from ALS patients [9, 13]. Moreover, an autopsy case of an ALS patient, who died unexpectedly during a minor surgical procedure, revealed severe denervation and reinnervation changes demonstrated by electromyography, but there were no detectable changes in the corresponding spinal motor neuronal cell bodies [9, 14]. Threshold tracking, which measures axonal excitability, is an alternative electrophysiological technique that demonstrates early abnormalities in

ALS patients. An apparent increase in persistent Na+ current and a decrease in K+ conductance is observed in two ALS patients [9, 15, 16], and these changes are more prominent distally than proximally [9, 17]. Genetical studies of ALS also provide further evidence for the potential importance of axonal pathology in ALS [9, 18].

4.4 Protein Misfolding and Axonal Degeneration in Experimental Animal Models

Recent studies demonstrate that genetically engineered mice with misfolded protein accumulation display axonal degeneration phenotype [19].

One of the excellent examples is the knockout mouse of an essential autophagy gene, *Atg7*, whose alterations have also been observed in several neurodegenerative diseases [20, 21]. Ablation of panneuronal autophagy causes ubiquitin-p62 positive aggregation in neuronal cell body [20, 21]. Conditional knockout of *Atg7* in Purkinje cells initially causes cell-autonomous, progressive dystrophy (manifested by axonal swellings) and degeneration of the axon terminals [22]. Consistent with suppression of autophagy, no autophagosomes are observed in these dystrophic swellings [22]. Axonal dystrophy of mutant Purkinje cells proceeds with little sign of dendritic or spine atrophy, indicating that axon terminals are much more vulnerable to autophagy impairment than dendrites. This early pathological event in the axons is followed by Purkinje cell death. Furthermore, ultrastructural analyses of mutant Purkinje cells reveal an accumulation of aberrant membrane structures in the axonal dystrophic swellings, indicating that the autophagic machinery component Atg7 is required for membrane trafficking and turnover in the axons, and that impairment of axonal autophagy as a possible mechanism for axonal degeneration associated with neurodegeneration [22]. Accordingly, significant accumulation of ubiquitinated proteins is noted in Atg7-deficient brain, but their levels, especially insoluble ubiquitinated proteins, are lower than in Atg7-deficient liver, and formation of the inclusion is found in restricted groups of neurons. Several ubiquitin-positive aggregates are recognized in Atg7-deficient brain regions in the presence of mild neuronal loss [22]. Direct degradation of aggregates/misfolded protein by autophagy is contradictory to the recent hypothesis that the generation of protein aggregates represents a protective mechanism [23]. However, the primary targets of autophagy are likely to be diffuse cytosolic proteins, not inclusion bodies themselves, suggesting that inclusion body formation in autophagy-deficient cells is an event secondary to impaired general protein turnover [23]. However, it is still possible that misfolded proteins in soluble or oligomeric states could be preferentially recognized by autophagosomal membranes, which might also be mediated by ubiquitin–p62–LC3 interactions [23, 24].

A recent study also showed that axonal degeneration is relevant to autophagy caused by protein mislocalization [25]. Adaptor protein-4 (AP-4) is a member of the adaptor protein complexes, which control vesicular trafficking of membrane proteins. Although AP-4 has been suggested to contribute to basolateral sorting

in epithelial cells, its function in neurons is unknown. A recent study showed that disruption of the gene encoding the β subunit of AP-4 resulted in increased accumulation of axonal autophagosomes, which contained alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors and transmembrane AMPA receptor regulatory proteins (TARPs), in axons of hippocampal neurons and cerebellar Purkinje cells both *in vitro* and *in vivo* [25]. AP-4 indirectly associates with the AMPA receptor via TARPs, and the specific disruption of the interaction between AP-4 and TARPs causes the mislocalization of endogenous AMPA receptors in axons of wild-type neurons. These results indicate that AP-4 may regulate proper somatodendritic-specific distribution of its cargo proteins, including AMPA receptor-TARP complexes and that protein mislocalization may disturb the autophagic pathway(s) in neurons [25].

4.5 Therapeutic Approaches to Treat Neuronal Dysfunction by Axonal Protection

4.5.1 Axonal Regeneration

From previous studies showing that neuronal dysfunction, which may morphologically reflect axonal degeneration by misfolded proteins, precedes neuronal cell death, we hypothesized that axonal regeneration may protect axons from degeneration and have therapeutic effects against neuronal dysfunction in neurodegeneration [26]. We have tested this hypothesis using anti-LINGO-1 antagonists in experimental PD models induced by either oxidative (6-hydroxydopamine) or mitochondrial (MPTP) toxicity [25]. LINGO-1 is the nervous system-specific leucine-rich repeat Ig-containing protein, and associated with the Nogo-66 receptor (NgR) complex and is endowed with a canonical EGF receptor (EGFR)-like tyrosine phosphorylation site, playing a critical role as an inhibitor of axonal regeneration (Fig. 4.1) [27, 28]. LINGO-1 antagonists, which block signal transduction of LINGO-1 complex (Fig. 4.2) [28], include decoy protein LINGO-1-Fc, Lenti-virus-dominant negative LINGO-1, and anti-LINGO-1 blocking antibody. We examined the role of LINGO-1 in cell damage responses of dopaminergic neurons. In LINGO-1 knockout mice, dopaminergic neuronal survival is increased and behavioral abnormalities are reduced compared with wild-type ones. This neuroprotection is accompanied by increased Akt phosphorylation [26]. Similar *in vivo* neuroprotective effects on midbrain dopaminergic neurons are obtained in wild-type mice by blocking LINGO-1 activity using LINGO-1-Fc protein which inhibit LINGO-1 function. Neuroprotection and enhanced neurite growth are also demonstrated for midbrain dopaminergic neurons *in vitro* [26]. LINGO-1 antagonists improve dopaminergic neuronal survival in response to MPTP in part by mechanisms that involve activation of the EGFR/Akt signaling pathway through a direct inhibition of the binding LINGO-1 to EGFR (Fig. 4.3) [26]. LINGO-1 is also upregulated in compromised, probably dysfunctional, neurons in spinal cord injury [29] or kainic acid injection

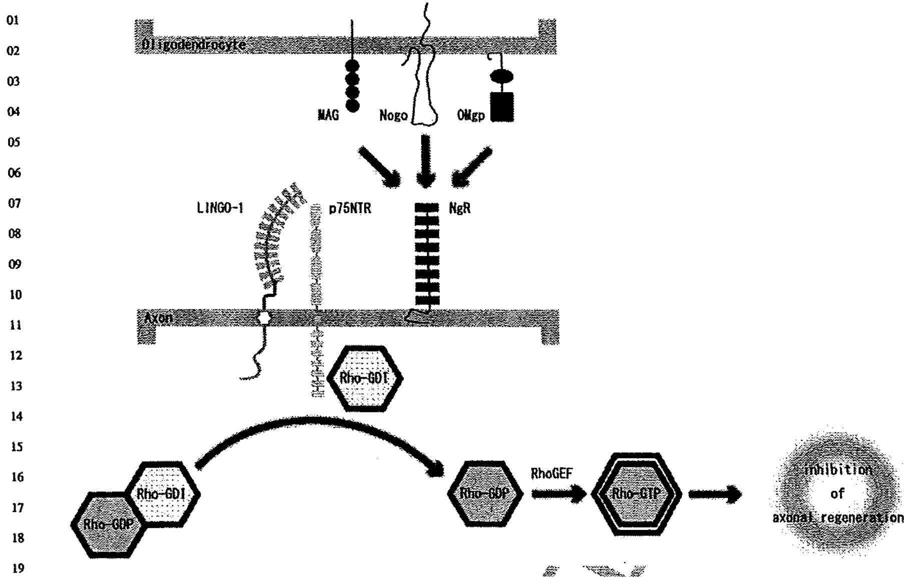

Fig. 4.1 Molecular signaling of Nogo-66 receptor (NgR) complex. The potential role of LINGO-1 is revealed as a component of NgR complex, which is comprised of NgR and p75 neurotrophin receptor (p75NTR) or an orphan TNF receptor Taj/Troy [27, 28]. Activated p75NTR binds the RhoA-GTP dissociation inhibitor (Rho-GDI), thus enabling RhoA activation via the exchange of GDP for GTP, and inhibits axonal regeneration upon binding to inhibitory molecules such as myelin-associated glycoprotein (MAG), oligodendrocyte myelin glycoprotein (OMgp), and Nogo-66 (Nogo) expressed in oligodendrocytes[40, 41]

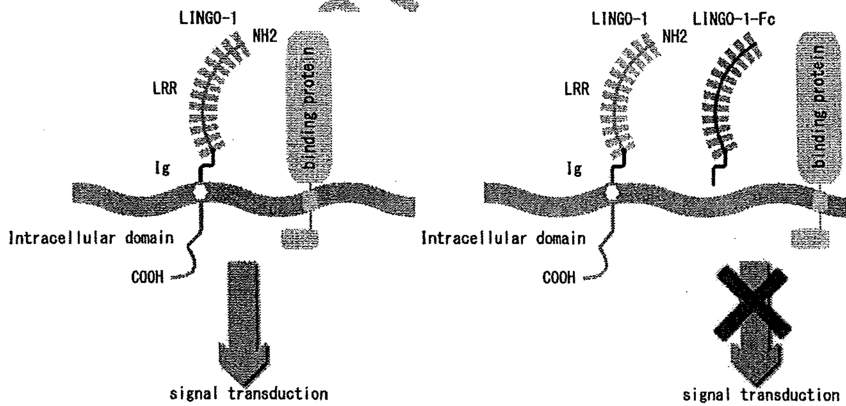

Fig. 4.2 Functional mechanisms of LINGO-1 antagonist(s). LINGO-1-Fc, one of LINGO-1 antagonists, is the soluble, truncated form of LINGO-1, and inhibits LINGO-1 modulating signaling transduction by inhibiting LINGO-1 to bind its binding protein(s) [26, 28]

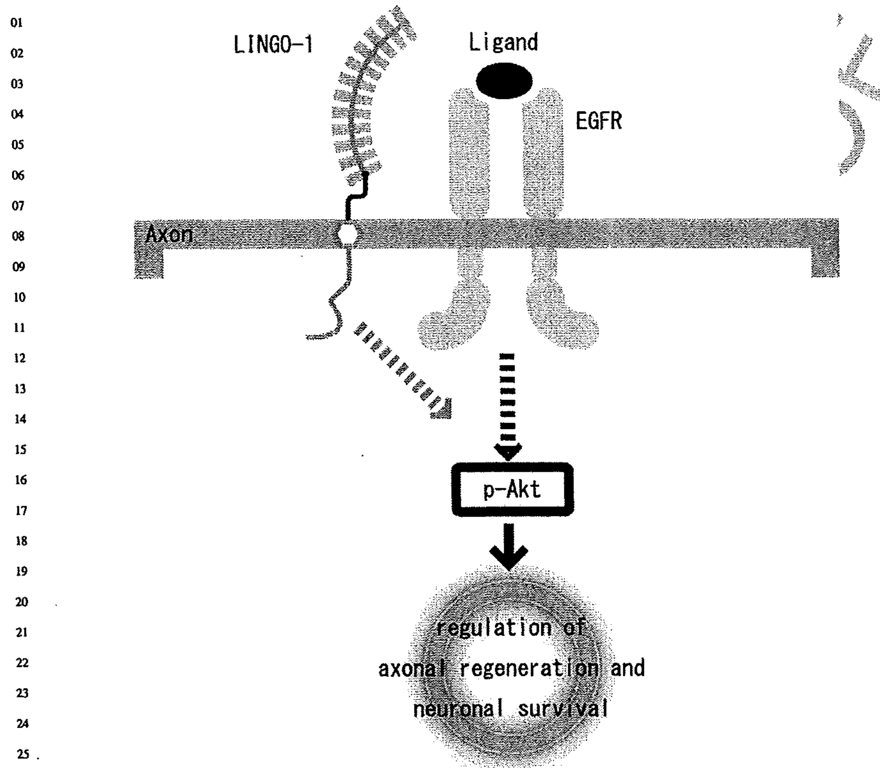

Fig. 4.3 LINGO-1 effect on EGF receptor (EGFR). LINGO-1 binds EGFR, and regulates EGFR expression level, leading to control axonal regeneration and neuronal survival via phosphorylation of Akt [26, 28]

[30]. We found that LINGO-1 expression is elevated in compromised, dysfunctional neurons including in the substantia nigra of PD patients compared with age-matched controls and in animal models of PD after neurotoxic lesions [25]. These results show that inhibitory agents of LINGO-1 activity can protect dopaminergic neurons from degeneration caused by PD. It is necessary to test whether LINGO-1 inhibition of function has protective effects on genetic PD models and/or other neurodegenerative disease models in the future.

4.5.2 Anti-Wallerian Degeneration

Axonal degeneration in "dying back" disorders seems to be different from Wallerian degeneration which is triggered by focal lesion (Fig. 4.4) [19]. However, apparent differences in the directionality of degeneration have been controversial [19].

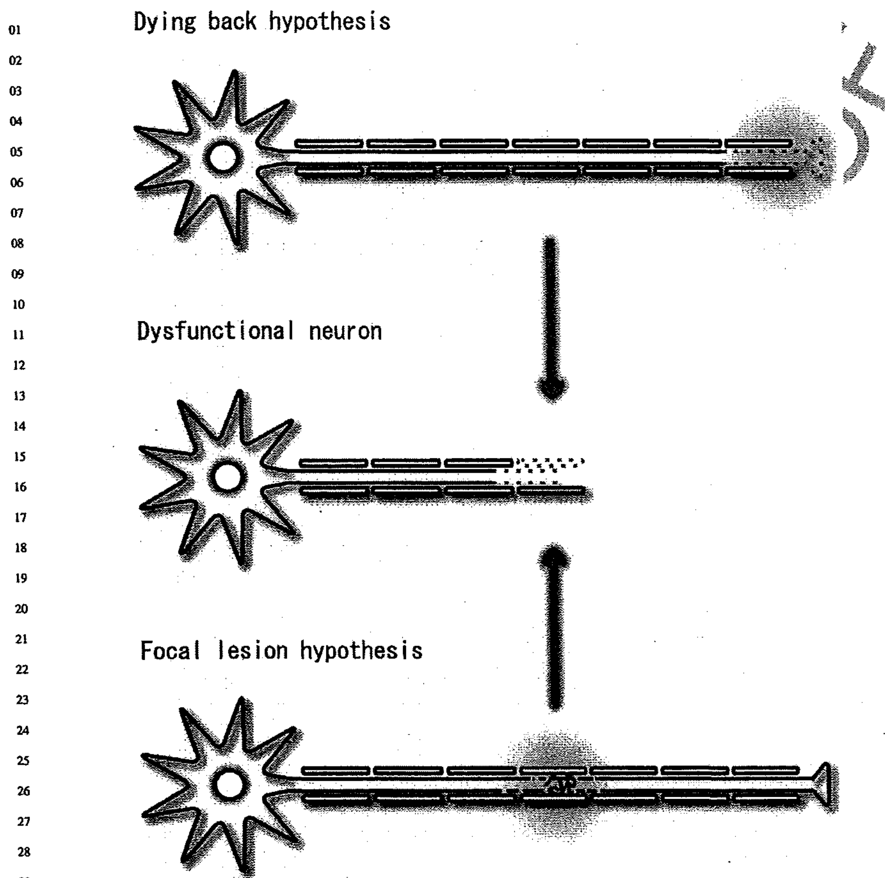

Fig. 4.4 "Dying back" and focal lesion models of axonal degeneration/dysfunction. The centripetal axonal degeneration in neurodegenerative disease may be caused either by the "dying back" process or by repetitive Wallerian degeneration from focal lesion(s) [19]

Wallerian degeneration is a simple experimental model of axonal degeneration, in which the distal stump of an injured axon degenerates rapidly after a reproducible latent phase [19]. In Wallerian degeneration slow (*WldS*) mice, Wallerian degeneration in response to axonal injury is delayed because of a mutation that results in overexpression of a chimeric protein (WldS) composed of the ubiquitin assembly protein Ufd2a and the nicotinamide adenine dinucleotide (NAD) biosynthetic enzyme Nmnat1 [31]. With the discovery of the *Wld*S mouse, the hypothesis could be tested. In *WldS* mice, injury-induced Wallerian degeneration is delayed ~tenfold (for 2–3 weeks) by a dominant mutation that acts intrinsically in neurons. In crossbreeding with progressive motor neuronopathy (*pmn*) mice and myelin protein zero (P0) null mutants, a model of Charcot-Marie-Tooth disease, WldS significantly

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delayed axonal degeneration [19]. In the central nervous system, WldS also protects against both genetic and toxic insults. Some nigrostriatal axons, which degenerate in PD, are spared and remain functional after 6-hydroxydopamine lesions in *WldS* mice [19, 32]. Axonal spheroids, which are presumably composed by misfolded protein(s) accumulation, are reduced in number in the gracile tract of mice with gracile axonal dystrophy (*gad*), deficient in ubiquitin carboxyterminal hydrolase L1 (UCHL1) crossed with *Wld*S mice [19, 33]. Not all axonal degeneration is delayed by WldS. WldS have modest effect on the ALS model mice with protection of the terminal axon only at its early stage [34, 35]. The failure to protect axons under certain circumstances indicates the existence of multiple axonal degeneration mechanisms. WldS may have protective effect(s) in rapidly degenerative or acute disorders.

4.5.3 Autophagy Enhancement

It is reasonable to assume that autophagy could represent a therapeutic target for axonal degeneration because the deletion of essential components of autophagy causes axonal degeneration, and relevance(s) of autophagy with degeneration are observed in several neurodegenerative diseases [23]. Autophagy enhancement by the regulatory protein kinase complex Target of Rapamycin (TOR) inhibitors such as rapamycin and its analogue CCI-779 protects against neurodegeneration seen in polyglutamine disease models in Drosophila and mice [23, 36]. A screened small molecule enhancers of rapamycin improve the clearance of mutant huntingtin and α-synuclein, and protect against neurodegeneration in a fruit-fly HD model [23, 37]. These results provide us with the evidence supporting autophagy enhancement as a therapeutic strategy against the toxicity of misfolded proteins in neurodegenerative diseases.

Tsc2, also known as tuberin, is a GTPase activating protein that regulates the G protein Rheb, an activator of mTOR (mammalian Target of Rapamycin) [38]. Tuberous sclerosis is a single-gene disorder caused by heterozygous mutations in the *TSC1* or *TSC2* genes and is frequently associated with mental retardation, autism and epilepsy [38]. Even individuals with tuberous sclerosis and a normal intelligence quotient are commonly affected with specific neuropsychological problems, including long-term and working memory deficits [38]. Mice heterozygous for the deletion of the *Tsc2* gene in *Tsc2*(+/−) mice show deficits in learning and memory [38]. A recent study showed that hyperactive hippocampal signaling led to abnormal long-term potentiation in the CA1 region of the hippocampus and consequently to deficits in hippocampal-dependent learning in TSC mice [38]. Moreover, a brief treatment with the mTOR inhibitor rapamycin in adult mice rescues not only the synaptic plasticity, but also the behavioral deficits in this animal model of tuberous sclerosis, demonstrating that treatment with mTOR antagonists ameliorates cognitive dysfunction in the TSC mice model [38]. Autophagy may be included in axon and/or synaptic dysfunction in degeneration via the mTOR signaling pathway(s).

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