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Review

Cbln and C1q family proteins – New transneuronal cytokines

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Abstract. The C1q family is characterized by a C-terminal conserved global C1q domain, which is structurally very similar to the tumor necrosis factor homology domain. Although some C1q family members are expressed in the central nervous system, their functions have not been well characterized. Cbln1, a member of the Cbln subfamily of the C1q family, is predominantly expressed in cerebellar granule cells. Interestingly, Cbln1 was recently shown to play two unique roles at excitatory synapses formed between cerebellar granule cells and Purkinje cells: the formation and stabilization of synaptic contact, and the control of functional synaptic plasticity by regulating the postsynaptic endocytosis pathway. Since other Cbln subfamily members, Cbln2–Cbln4, are expressed in various regions of developing and mature brains, Cbln subfamily proteins may generally serve as a new class of transneuronal regulators of synapse development and synaptic plasticity in various brain regions.

Keywords. Cerebellin, cerebellum, synapse formation, synaptic plasticity, tumor necrosis factor, Purkinje cell, granule cell.

Introduction

C1q is the target recognition protein of the classical complement pathway. Its C-terminal globular domain (gC1q) forms a trimer and recognizes a broad range of ligands, such as pentraxins, IgG, IgM, β-amyloid fibrils, lipopolysaccharides, porins from Gram-negative bacteria, and phospholipids. The gC1q signature domain has also been identified in a variety of non-complement proteins grouped together as the C1q family. X-ray crystallographic studies further revealed that the gC1q domain of a few members of the C1q family had a compact jelly-roll β-sandwich fold (Fig. 1), similar to the tumor necrosis factor (TNF) homology domain (THD) of the multifunctional TNF ligand family. Although some C1q family members are selectively expressed in the central nervous system (CNS), their functions have not been well characterized. Cbln1, which belongs to a Cbln subfamily of the C1q family, is predominantly expressed in cerebellar granule cells. Interestingly, Cbln1 was recently shown to play two unique roles at excitatory synapses formed between cerebellar granule cells and Purkinje cells: the formation and stabilization of synaptic contact, and the control of functional synaptic plasticity by regulating the postsynaptic endocytosis pathway [1]. Similarly, C1q was recently shown to be expressed transiently by postnatal neurons and to mediate synapse elimination in the CNS [2]. Thus, some C1q family proteins may serve as new cytokines that may regulate normal and abnormal brain functions. The aim of this review is to provide an update on the structure and functional aspects of the Cbln subfamily of the C1q family. For a more general overview of other C1q members, please refer to reviews elsewhere [3–6].

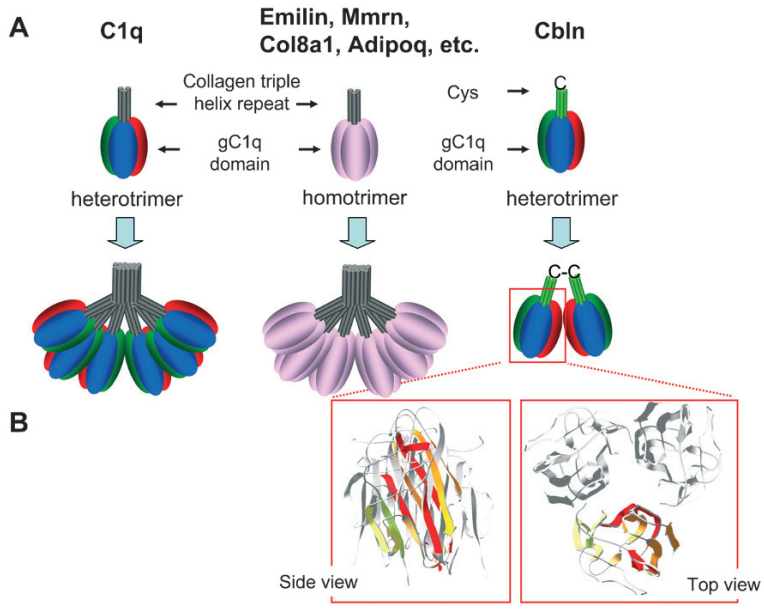

Figure 1. Structural organization of the C1q family proteins. (*A*) C1q (left) and Cbln (right) form heteromeric trimers, whereas other C1q family members, such as Emilin, Mmrn (multimerin), Col8a1 (collagen VIII), and Adipoq (adiponectin) form homomeric trimers. The N-terminal collagen triple helix repeat and cysteine residues facilitate the formation of a higher order complex. (*B*) A trimeric structural model of the gC1q domain of Cbln1 generated by the Swiss-PdbViewer (Swiss Institute of Bioinformatics, Geneva, Switzerland) using the structure of C1q (1pk6) as a template. Side and top views of predicted Cbln1 trimers are shown in the left and right panels.

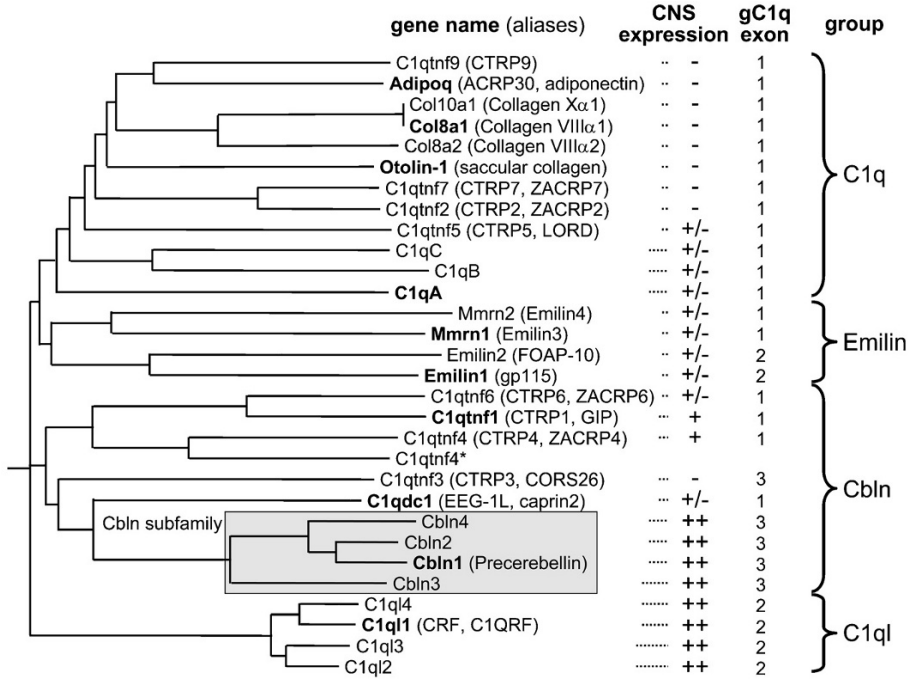

Figure 2. Phylogenetic tree of mouse C1q family members. A phylogenetic dendrogram was generated from ClustalW alignment of all mouse gC1q domain sequences using the PHYLIP program (The Biology Workbench 3.2, San Diego Supercomputer Center at University of California, San Diego). The gene name, its expression in the CNS as suggested by an analysis of the expressed sequence tag counts (National Center for Biotechnology Information, EST Profile Viewer), the numbers of exons encoding each gC1q domain, and the assignment of four groups of homology-related sequences are indicated on the right side of the phylogenetic tree. The Cbln subfamily of the Cbln group is indicated by a shaded box. Representative C1q family members whose domain organization is shown in Fig. 3 are indicated in bold.

General features of C1q family proteins and their expression in the CNS

In a pioneering review on the TNF and the C1q superfamily in 2002 [3], the TNF family was thought to comprise 18 genes encoding 19 transmembrane proteins, while the C1q family contained 13 members. Since then, many new proteins containing the C1q domain have been identified, and the C1q family, with a total of 32 members in humans and 29 in mice, is now larger than the TNF family (still 19 members). Phylogenetic analysis of the gC1q domain suggests that the mouse C1q family could be divided into four groups, each represented by a typical subfamily name: C1q, Emilin, Cbln, and C1ql (Fig. 2). Except for the Cbln subfamily of the Cbln group, the expression patterns of these proteins in the CNS have not been systemically characterized; however, public-domain databases at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov) indicate that the Cbln and C1ql subfamilies are highly expressed in the CNS (Fig. 2).

The overall structure of each C1q family member protein is quite variable, but the gC1q domain is highly conserved and always located at the extreme C terminus (Fig. 3). Thus, member proteins are thought to form trimers, composed of heteromers (C1q and Cbln), homomers (Adipoq, Mmrn1, Mmrn2, Emilin, Col10a1, Col8a1 and Col8a2), or unknown composition (C1ql and others) (Fig. 1). All proteins except for C1qdc1 have signal sequences without any transmembrane domains. In addition, most members contain a collagen-like sequence, which forms a triple helix, at the N terminus of the gC1q domain. Thus, a unit trimer formed by the gC1q domain is further organized into a higher order multimeric complex (Fig. 1). For members that do not contain collagen-like sequences, other structures are thought to be involved in the formation of higher order multimeric complexes: EMI [7] and coiled-coil domains [8] are used in Multimerin (Mmern), coiled-coil domains in C1qdc1, a second gC1q domain in C1qtnf4, and cysteine residues in Cbln [9] (Fig. 3). As a result, six C1qA-C1qB-C1qC trimers assemble into a complex containing 18 polypeptide chains, but the number of trimers in a complex may vary in other C1q family members. For example, adiponectin (Adipoq) could exist as a trimer, a dimer of trimers, or higher order trimers; the oligomeric status of adiponectin is reported to be more closely associated with insulin sensitivity and metabolic syndrome than the total amount of adiponectin [10].

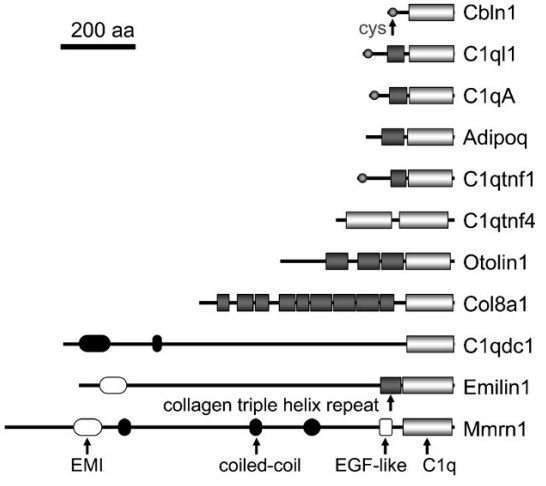

Figure 3. Domain organization of the representative C1q family members. The C-terminal gC1q signature domain (light gray boxes) is thought to form a trimer complex. The N-termini triple-helical Gly-X-Y collagen repeat (dark gray boxes) is thought to be involved in the higher order multimerization of trimers. For C1q members that lack the collagen repeat domain, such as Cbln, C1qtnf4 (CTRP4, ZACRP4), Mmrn (multimerin), and C1qdc1 (EEG-1L, caprin2), cysteines, a second gC1q domain, EMI domains (white circles), or coiled-coil domains (black circles) are thought to be involved in higher-order multimerization.

Cbln subfamily

Genes and evolutional aspects. The Cbln subfamily consists of four members, Cbln1–Cbln4 (Fig. 2); the full-length amino acid sequence is 57–79 % similar to, and 71–86 % identical to each other. Their genes are located at different chromosomes: Cbln1, 8C3; Cbln2, 18E4; Cbln3, 14C3; Cbln4, 2H3 in mice; and Cbln1, 16q12.1; Cbln2, 18q22.3; Cbln3, 14q12; Cbln4, 20q13.13-q13.33 in humans. So far, no spontaneous mutations in these genes have been associated with diseases or abnormal phenotypes in mice and humans. It was previously pointed out that the C1q and TNF family proteins had similar gene structures: their gC1q or THD domains are each encoded within one exon [4]. In contrast, the gC1q domains of Cbln subfamily members are encoded by three exons (Fig. 2). Similarly, the gC1q domains of the C1ql subfamily and a few C1q proteins span several exons (Fig. 2). Thus, the C1q and TNF family proteins may not simply have diverged from a single common precursor molecule. The phylogenetic analysis of the amino acid usage of the gC1q domain also supports the view that Cbln and C1ql subfamilies evolved differently from the rest of the C1q family proteins (Fig. 2).

The C1q family proteins have an ancient evolutionary history. They are present in some bacteria (*Bacillus cereus*) and echinoderms (*Strongylocentrotus purpuratus*), but are completely absent in plants (*Arabidopsis thaliana*), yeasts (*Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*), nematodes (*Caenorhabditis elegans*), and insects (*Drosophila melanogaster*). All Cbln proteins are highly conserved among mammals. For example, 96–100 % of the amino acid sequences of Cbln1–Cbln4 are identical between mice and humans. In contrast, Cbln3, or at least its closely related proteins, seem to be absent in frog (*Xenopus laevis*) and fish (*Danio rerio*). In birds (*Gallus gallus*), only one protein related to Cbln4 could be identified. These findings suggest that Cbln4 may be the prototype of the Cbln subfamily and that Cbln3 may be the last member that emerged in mammals.

Expression. Cbln1 mRNA is highly expressed in granule cells and deep nuclei in the cerebellum [11]. Outside the cerebellum, Cbln1 mRNA is enriched in a few regions, including the mitral layer of the olfactory bulb, the retrosplenial granular cortex, and certain thalamic nuclei. The expression of Cbln2 mRNA is widespread in the CNS, with higher levels in the olfactory bulb, cerebral cortex, certain thalamic and hypothalamic nuclei, superior and inferior colliculi, and some brainstem nuclei. In contrast, the expressions of Cbln3 and Cbln4 mRNAs are restricted to cerebellar granule cells and certain thalamic nuclei,

respectively [11]. During development, Cbln1, 2, and 4 mRNAs are expressed as early as embryonic day 13 and show transient up-regulation during the perinatal period. On the other hand, Cbln3 mRNA is first detected on postnatal day 7 and is strikingly up-regulated around postnatal day 14 [11]. Thus, distinct Cbln subtypes are expressed in various regions of developing and mature brains.

Different Cbln subtypes are often coexpressed in some nuclei and regions of the adult brain. For example, Cbln1 and Cbln2 mRNAs are coexpressed in the retrosplenial granular cortex and lateral habenula, Cbln1 and Cbln3 mRNAs in the cerebellar granular layer, and Cbln1 and Cbln4 mRNAs in the entorhinal cortex [11]. As Cbln family proteins form heteromeric complexes with each other (Fig. 1), such combinatory expression may give rise to further functional heterogeneities.

Interestingly, none of the four Cbln mRNAs have been detected in several principal neurons, such as cerebellar Purkinje cells, hippocampal granule and pyramidal cells, and caudate-putamen, which send information outside the local circuit. In contrast, dentate granule cells and hippocampal pyramidal cells receive major inputs from the entorhinal cortex, which highly expresses Cbln1 and Cbln4 mRNAs. Similarly, Purkinje cells receive massive inputs from cerebellar granule cells that express Cbln1 and Cbln3 mRNAs, and the caudate-putamen are innervated by inputs from thalamic nuclei, which highly express Cbln1 and Cbln2 mRNAs. Thus, Cbln mRNAs may be generally expressed in presynaptic neurons that send numerous axons to principal neurons and function at such synapses. Furthermore, since Cbln1, 2, and 4 mRNAs are expressed as early as embryonic day 13, when no synapses have yet formed, they may also be involved in other nonsynaptic functions.

The protein expression patterns of the Cbln subfamily are less clear. Cbln1-like immunoreactivity was originally reported to be predominantly localized at dendritic spines and the somata of cerebellar Purkinje cells [12–14], which do not express Cbln1 mRNA. Thus, Cbln1 proteins synthesized in granule cells may be rapidly released and taken up by postsynaptic Purkinje cells. In contrast, in a very recent report, Cbln1-like immunoreactivity was not observed in Purkinje cells but was localized in the lysosomal compartment of cerebellar granule cells [15]. Since Cbln1 undergoes extensive proteolysis [9], like several other C1q family proteins, the discrepant results may reflect different antibodies recognizing various proteolytic fragments of Cbln1.

Function. The cerebellum-specific hexadecapeptide "cerebellin" was originally identified more than 20 years ago [16]. Later, it became clear that cerebellin was derived from Cbln1, a member of the C1q family [17]. However, its function remained unclear until mice lacking the *cbln*1 gene (*cbln1*$^{-/-}$ mice) were analyzed [1]. *cbln1*$^{-/-}$ mice were ataxic, having a markedly impaired performance on the rotorod test (Fig. 4). Nevertheless, the cerebellum and other brain regions of *cbln1*$^{-/-}$ mice showed no overt neuroanatomical anomalies that could account for the ataxic behavior. Detailed electrophysiological and electron microscopic analyses of *cbln1*$^{-/-}$ mice have revealed that Cbln1 plays two crucial roles at parallel fiber (PF; an axon of a granule cell)–Purkinje cell synapses (Fig. 4) [1]. First, the number of PF–Purkinje cell synapses is markedly reduced, so that as many as 80 % of the spines lose synaptic contact with PFs in adult *cbln1*$^{-/-}$ cerebella. In addition, in the remaining PF–Purkinje cell synapses in *cbln1*$^{-/-}$ mice, the postsynaptic densities (where glutamate receptors and their anchoring proteins are clustered) were frequently longer than the presynaptic active zones (where glutamate-containing synaptic vesicles preferentially fuse), whereas the length of the active zones completely matched that of the postsynaptic densities in wild-type excitatory synapses. This unique morphological phenotype suggests that Cbln1 may function as an adhesive molecule regulating matching and the maintenance of pre- and postsynaptic structures at PF–Purkinje cell synapses.

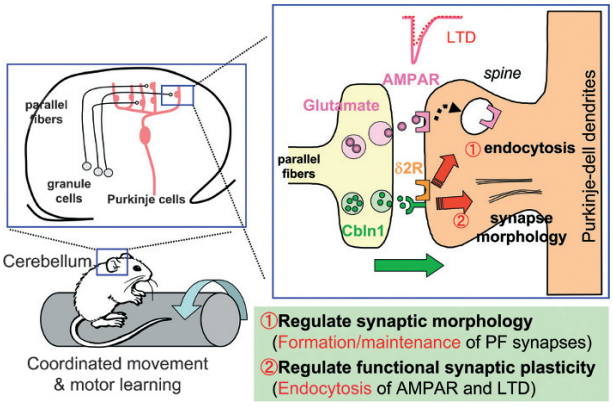

Figure 4. Two unique functions of Cbln1. A Cbln subfamily member Cbln1 is preferentially expressed in granule cells in the cerebellum. *cbln1*$^{-/-}$ mice are ataxic, having markedly impaired performance on the rotorod test (left). Although no overt neuroanatomical anomalies are found in the cerebellum, detailed electrophysiological and electron microscopic analyses of *cbln1*$^{-/-}$ mice revealed that Cbln1 plays two crucial roles at parallel fiber (axon of the granule cell, PF)–Purkinje cell synapses: the formation and stabilization of PF–Purkinje cell synaptic contact and control of functional synaptic plasticity (LTD: long-term depression) by regulating the postsynaptic endocytosis pathway of AMPA receptors (right). These abnormalities in *cbln1*$^{-/-}$ mice are shared by mice that lack orphan δ2 glutamate receptors (δ2R), which are predominantly expressed in Purkinje cells.

Simultaneous activation of PFs and climbing fibers normally induces the long-term depression (LTD) of synaptic transmission at PF–Purkinje cell synapses; LTD is a form of synaptic plasticity thought to underlie motor coordination and information storage in the cerebellum [18]. The second crucial role played by Cbln1 is the induction of LTD; LTD is completely impaired in *cbln1*$^{-/-}$ mice (Fig. 4) [1]. It is thought that the concurrent activation of climbing fibers and PFs increases intracellular Ca^{2+} levels and activates protein kinase Cα in Purkinje cells [19], which phosphorylates GluR2 subunits of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)-type glutamate receptors [20]. Upon phosphorylation, GluR2 subunits are released from the anchoring protein GRIP and are captured by another anchoring protein PICK1 [21], which initiates the endocytosis of AMPA receptors in Purkinje cells [22, 23]. Because cerebellar LTD occurs solely in postsynaptic Purkinje cells, Cbln1 expressed and released from granule cells may transneuronally regulate some steps causing the activity-dependent endocytosis of AMPA receptors in Purkinje cells during LTD (Fig. 4).

Interestingly, all the unique ultrastructural and electrophysiological abnormalities in *cbln1*$^{-/-}$ mice are shared by mice that lack orphan δ2 glutamate receptors (GluRδ2) [24], which are predominantly expressed in Purkinje cells (Fig. 4). Although GluRδ2 by itself does not bind to glutamate or directly participate in neurotransmission, GluRδ2-null mice exhibit impaired cerebellar LTD and severe ataxia [25, 26]. Furthermore, in adult GluRδ2-null cerebellum, approximately 40% of the spines on distal dendrites of Purkinje cells remain uninnervated by PFs, and a mismatch is frequently observed between the lengths of the postsynaptic densities and the apposing presynaptic active zones in the remaining PF synapses [27, 28]. These results suggest that GluRδ2, which is located in PF–Purkinje cell postsynapses, and Cbln1, which is expressed in granule cell presynapses, engage in a common signaling pathway or process crucial for synapse formation/maintenance and plasticity. Indeed, mice that lack both GluRδ2 and Cbln1 do not show an additive phenotype but rather are similar to mice lacking only GluRδ2, a finding implying that the two gene products function in a common mechanism or pathway, rather than acting entirely independently [1].

The function of the originally identified peptide "cerebellin" remains unclear, although it has been reported to stimulate the secretion of nor-epinephrine in adrenal glands [29, 30]. However, Cbln1 mRNA was not detected in normal adrenal glands using a Northern blot analysis or by reverse transcription followed by polymerase chain reaction [11, 17, 31]. Similarly, public-domain databases at the National Center for Biotechnology Information (UniGene) indicate that Cbln subfamily member mRNAs are not expressed in adrenal glands. It is still possible that a very small number of cells or axons may express Cbln1 in adrenal glands and that its degradation product, cerebellin, may locally regulate the secretion of nor-epinephrine. The expression of Cbln1 mRNA in pituitary glands and pineal glands (UniGene) suggests a putative regulatory role in secretory vesicles. Alternatively, exogenously applied cerebellin may simply cross-react with unrelated receptors in adrenal glands.

Processing and regulation. Cbln1 is secreted from cerebellar granule cells and heterologous cells transfected with Cbln1 cDNA as an N-linked glycoprotein [1]. It forms a trimer *via* its C-terminal gC1q domain and a hexamer consisting of two trimers connected *via* N-terminal disulfide bonds (Fig. 1) [9]. Similarly, Cbln4 is secreted as a glycoprotein from heterologous cells. Although Cbln2 was originally reported as a non-secretory protein [32, 33], it is secreted from heterologous cells, probably with less efficiency than Cbln1 and Cbln4 [34]. In contrast, despite the presence of a signal sequence, Cbln3 is not secreted at all but retained in the endoplasmic reticulum (ER)/cis-Golgi [33, 34] in heterologous cells. When Cbln1 and Cbln3 are coexpressed in heterologous cells, a proportion of the Cbln1 protein is retained in the ER/cis-Golgi; conversely, some Cbln3 proteins are secreted together with Cbln1. Similarly, in wild-type granule cells expressing Cbln1 and Cbln3, Cbln3 proteins are partially secreted, while Cbln3 is completely degraded in *cbln1*$^{-/-}$ granule cells, probably through an ER-associated degradation mechanism (Fig. 5) [33, 34]. These results indicate that heteromer formation between Cbln1 and Cbln3 in cerebellar granule cells may modulate each other's trafficking and signaling pathways. We hypothesize that Cbln3 may have emerged in mammals to fine-tune the function of Cbln1 in the cerebellum.

Although most C1q family proteins have signal sequences and are secreted from cells, the gC1q domain has also been suggested to function inside cells [35]. For example, C1qtnf1 (CTRP1, GIP) is likely a membrane protein that associates with G protein–-coupled receptors on plasma membranes [36]. C1qdc1 (EEG-1L, caprin) is the only C1q family protein lacking a signal sequence and exists instead as a cytosolic soluble protein [37]. Like Cbln3, C1ql1 (CRF, C1QRF) has a signal sequence but is retained in the ER or cis-Golgi [38]. Thus, in addition to serving as a regulator of Cbln1 secretion, the gC1q domain of Cbln3 may function inside cells.

It is not completely clear why Cbln3 is retained in the ER/cis-Golgi. Since the gC1q domain of Cbln3 did not

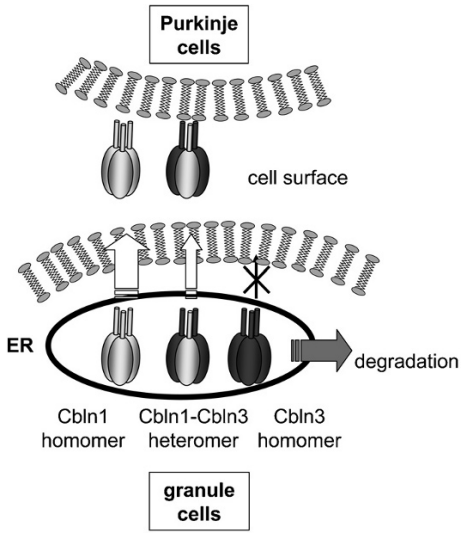

Figure 5. Model of the interaction between Cbln1 and Cbln3. Cbln1 homomers are secreted efficiently and reach Purkinje cells, whereas Cbln3 homomers are retained in the endoplasmic reticulum (ER) and degrade rapidly in granule cells. Cbln1 also forms a heteromer with Cbln3, but this may take longer than the formation of a homomer and the resulting heteromer may not be secreted efficiently.

interact with each other in a yeast two-hybrid assay, it was proposed that unstable trimers formed by Cbln3 may be trapped by the ER quality control mechanism [33]. Indeed, various mutations within the gC1q domain of Col10a (collagen X) in certain forms of Schmid metaphyseal chondrodysplasia [39] cause the retention of mutant proteins in the ER. However, unlike in yeast, in mammalian heterologous cells Cbln3 could form a homomeric complex [34]. In addition, Cbln3 lacking the N-terminal domain was secreted from heterologous cells [34]. Furthermore, when the N terminus of Cbln3 was replaced with that of Cbln1, the chimeric protein (NT-Cbln1-Cbln3) was secreted into the medium [34]. Therefore, although the gC1q domain of Cbln3 may be involved in its retention in the ER/cis-Golgi, the N terminus of Cbln3 is also likely to play a crucial role in the trafficking of Cbln3.

The "cerebellin" peptide is associated with the synaptosomal fraction [16] and is released upon depolarization in a calcium-dependent manner [40]. A related family member, TNF-α, is cleaved from membrane-bound pro-TNF-α through the action of TNF-α-converting enzyme (TACE) [41]. Interestingly, the region preceding the gC1q domain of Cbln subfamily proteins (SVRSGS in Cbln1 and Cbln2 and SVRAAN in Cbln4) is similar to the corresponding region of pro-TNF-α (AVRSSS) recognized by TACE. However, since full-length Cbln1 is released into a medium from cultured granule neurons and heterologous cells [9], proteolytic cleavage is not required for the secretion of Cbln1. Since Cbln1-like immunoreactivity is localized in the lysosomal compartment of cerebellar granule cells [15], proteolysis by TACE-like enzymes may represent a mechanism to regulate the amount of Cbln1 secretion in presynaptic neurons. Further studies are warranted to clarify how Cbln subfamily proteins are released from neurons.

Future direction. Although great progress has been made in understanding Cbln signaling during the past several years, much remains to be learned about its mechanism of signaling. For example, we still do not know how Cbln proteins are secreted and how they are removed from synapses. A key question is what receptors Cbln proteins bind and what intracellular signaling pathways are activated. In the TNF family, the THD domain, which is equivalent to the gC1q domain of the C1q family, interacts with each specific receptor [3]. Similarly, the gC1q domain of adiponectin is recognized by its receptor AdipoR [42]. However, because the gC1q domain could interact with a broad range of proteins, identification of specific receptors is not straightforward. Indeed, many receptor candidates have been reported for C1q [43] and adiponectin [42, 44]. Striking phenotypic similarities shared by *cbln1*$^{-/-}$and GluRδ2-null mice suggest that GluRδ2, which is located in the PF–Purkinje cell postsynapses, may be a part of the receptor complex for Cbln1 in Purkinje cells. Since Cbln1 is found in brain regions where GluRδ2 is not expressed, Cbln1 may bind to other receptors in such regions.

Concluding remarks

Cbln1, a Cbln subfamily member preferentially expressed in cerebellar granule cells, plays two novel roles at PF–Purkinje cell synapses: formation and stabilization of PF–Purkinje cell synaptic contact and control of functional synaptic plasticity by regulating the postsynaptic endocytosis pathway of AMPA receptors. Cbln3, which is coexpressed in cerebellar granule cells, may serve as a regulatory protein by forming heteromeric complex with Cbln1. Other Cbln subfamily members, Cbln2–Cbln4, are expressed in various regions of developing and mature brains. In addition, all members of the Cbln subfamily form heteromeric complexes with each other. Therefore, all Cbln subfamily proteins may also serve as trans-neuronal regulators of synapse development and synaptic plasticity in various brain regions, probably by forming heteromeric complexes.

Very recently, C1q was shown to be expressed transiently by postnatal neurons and to mediate synapse elimination during development [2]. Other

C1q family proteins, such as the C1ql subfamily, are also expressed in various regions of the CNS. Furthermore, although adiponectin (Adipoq) and hibernation proteins (a C1q family protein expressed in mammalian hibernators), are produced in peripheral tissues, they have recently been shown to exert unique functions in specific brain regions [45, 46]. Therefore, an understanding of Cbln subfamilies' functions will also provide new insights into the roles of C1q family proteins as new cytokines that may regulate normal and abnormal brain functions, thus permitting the development of novel approaches for the treatment of particular neurological disorders.

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