Inhibition of Protein Misfolding/Aggregation Using Polyglutamine Binding Peptide QBP1 as a Therapy for the Polyglutamine Diseases

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Abstract Protein misfolding and aggregation in the brain have been recognized to be crucial in the pathogenesis of various neurodegenerative diseases, including Alzheimer's, Parkinson's, and the polyglutamine (polyQ) diseases, which are collectively called the “protein misfolding diseases”. In the polyQ diseases, an abnormally expanded polyQ stretch in the responsible proteins causes the proteins to misfold and aggregate, eventually resulting in neurodegeneration. Hypothesizing that polyQ protein misfolding and aggregation could be inhibited by molecules specifically binding to the expanded polyQ stretch, we identified polyQ binding peptide 1 (QBP1). We show that QBP1 does, indeed, inhibit misfolding and aggregation of the expanded polyQ protein *in vitro*. Furthermore overexpression of QBP1 by the crossing of transgenic animals inhibits neurodegeneration in *Drosophila* models of the polyQ diseases. We also introduce our attempts to deliver QBP1 into the brain by administration using viral vectors and protein transduction domains. Interestingly, recent data suggest that QBP1 can

also inhibit the misfolding/aggregation of proteins responsible for other protein misfolding diseases, highlighting the potential of QBP1 as a general therapeutic molecule for a wide range of neurodegenerative diseases. We hope that in the near future, aggregation inhibitor-based drugs will be developed and bring relief to patients suffering from these currently intractable protein misfolding diseases.

Keywords Polyglutamine disease · Neurodegeneration · QBP1 · Protein aggregation · Inhibitor peptide · Therapy

Introduction

Recent accumulating evidence has indicated that many neurodegenerative diseases including Alzheimer's disease (AD), Parkinson's disease (PD), the polyglutamine (polyQ) diseases, amyotrophic lateral sclerosis (ALS), and the prion diseases share a common pathomechanism involving protein misfolding/aggregation and their accumulation in the brain. Pathologic and biochemical studies have revealed that various protein depositions are found inside and outside of neurons in the diseased brains, such as senile plaques composed of amyloid- β in AD [1] and Lewy bodies composed of α -synuclein in PD [2]. Although the significance of these protein depositions on disease pathology long remained controversial, recent molecular genetics studies revealed that the mutations responsible for the inherited forms of these neurodegenerative diseases render the proteins to be prone to misfolding and aggregating, or lead to the overproduction of aggregation-prone proteins [3–5]. Furthermore, not only the inherited forms of these diseases, but the sporadic cases also exhibit similar protein accumulations in the brain, further indicating that misfolding and abnormal aggregation of proteins are crucial in the pathogenesis of these

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neurodegenerative diseases, which are, hence, collectively called the “protein misfolding diseases” [3–5].

In this review we will introduce our research towards establishing a therapy for the polyQ diseases by targeting protein misfolding and aggregation using polyglutamine binding peptide 1 (QBP1).

The Polyglutamine Diseases

The polyQ diseases are a group of neurodegenerative diseases caused by a common genetic mutation, namely an expansion (>40) of the CAG repeat encoding a polyQ stretch in each unrelated disease-causing gene [6, 7]. Nine diseases currently belong to this group, including Huntington’s disease, spinocerebellar ataxia type 1, 2, 3, 6, 7, and 17, dentatorubral pallidoluysian atrophy, and spinobulbar muscular atrophy (SBMA) [7]. In these diseases, progressive degeneration of neurons in brain areas specific for each disease occurs, causing various neurologic and psychiatric symptoms corresponding to each affected brain area.

Numerous sources of evidence has led to the consensus that the expanded polyQ stretch itself is the cause of the polyQ diseases based on a gain of toxic function mechanism. First, they are inherited in an autosomal dominant manner (except for SBMA). Second, the polyQ stretch is the only homologous sequence among the disease-causing proteins, which show no other functional or sequence similarity. Furthermore, the polyQ stretch is the major determinant of disease, as shown by a threshold between normal and disease of 35–40 repeats (except for spinocerebellar ataxia type 6), and a tight correlation between polyQ repeat length and the age of disease onset and severity. Third, loss-of-function mutations in the disease-causing gene of SBMA (androgen receptor gene) do not cause neurodegenerative disease. Finally, expression of an expanded polyQ stretch alone or even inserted into an unrelated protein is sufficient to cause neurodegeneration in various experimental animal models [8–11].

To study the molecular pathomechanisms of, and potential therapies for, the protein misfolding diseases, the polyQ diseases are the most suitable model as they possess the following characteristics. First, as they are determined almost solely by a monogenic mutation, it is relatively easy to establish genetic models, unlike the other diseases, which are caused by multiple genetic and environmental factors. Second, as there is a strong genotype–phenotype correlation, the severity of the experimental model can be manipulated as suitable for analysis. Our group has therefore been using the polyQ diseases as a model of the protein misfolding diseases, towards establishing a therapy for these diseases.

In the common molecular pathogenesis of the polyQ diseases, proteins with an expanded polyQ stretch become misfolded and undergo a toxic conformational transition to a β -sheet dominant monomer [12], then form oligomers/aggregates, and, subsequently, accumulate as inclusion bodies within neurons, eventually resulting in neurodegeneration [13–17]. Although therapeutic strategies against various cellular processes thought to be eventually impaired in the polyQ diseases have been investigated [16, 18], they have resulted in limited therapeutic effects, as numerous processes are affected by the expanded polyQ protein [19]. In contrast to the downstream effects, misfolding and aggregation of the expanded polyQ protein are ideal therapeutic targets as they are the most initial pathogenic events, and therefore their inhibition is expected to result in the suppression of a broad range of downstream pathogenic events [14, 15, 20–22] (Fig. 1). We therefore aimed to establish a therapy targeting misfolding and aggregation of the expanded polyQ protein.

Identification of QBP1

We hypothesized that misfolding/aggregation of the expanded polyQ protein could be inhibited by peptides binding specifically to the expanded polyQ stretch, as A β aggregation was shown to be inhibited by A β binding peptides [23]. Furthermore specific recognition of the expanded polyQ stretch, but not normal one by a peptide seemed feasible,

Fig. 1 Therapeutic target and effect of polyglutamine binding peptide 1 (QBP1). Proteins with an expanded polyglutamine (polyQ) stretch are prone to misfold, which then affects multiple cellular events, which will eventually lead to neurodegeneration. QBP1 inhibits the initial misfolding of the expanded polyQ protein, resulting in inhibition of a wide range of downstream pathogenic events and, hence, is able to suppress neurodegeneration

as a monoclonal antibody (1C2) was shown to bind selectively to expanded polyQ stretches, probably by detecting their unique structure [24]. We therefore screened for peptides that bind selectively to the expanded polyQ stretch by using the phage display technique [25]. Eleven amino acid combinatorial peptide libraries expressed on the surface of M13 phage were analyzed for their binding to a Q62 stretch by enzyme immunosorbent assay. The positive phage clones were then analyzed further for their selective binding to the expanded length Q62 compared with the normal length Q19. Six phage clones with greater binding affinity for Q62 compared with Q19 were identified, and the encoded peptide sequences were named polyQ binding peptide 1–6 (QBP1–6; Table 1). QBP1 (SNWKWWPGIFD), which showed the greatest differential binding affinity to the pathologic length polyQ compared with normal length polyQ, was chosen for further analysis.

We further characterized the binding properties of QBP1 to the expanded polyQ stretch by using the surface plasmon resonance technique. QBP1 was found to bind to Q62 with an equilibrium dissociation constant (K_d) of 5.7 μ M, while it did not bind significantly to either Q0 nor Q19 [26]. A scrambled sequence of QBP1 (SCR; WPIWSKGNDWF), however, did not show any binding affinity to Q62. These results confirm the striking property of QBP1 to bind selectively to the expanded polyQ stretch, but not normal one probably by recognizing a conformation that is present only in the expanded polyQ stretch.

QBP1 Inhibits the Toxic Conformational Transition and Aggregation of the Expanded PolyQ Protein

We next tested the effect of QBP1 on the conformation of the expanded polyQ protein. Using circular dichroism analyses we demonstrated that QBP1 inhibits the initial β -sheet

conformational transition of the expanded polyQ protein monomer [12] (Fig. 1; Table 2). Furthermore, we showed, by microinjection of different polyQ protein species into cultured cells, that this β -sheet conformational transition causes cytotoxicity. Recently, Carrion-Vazquez and colleagues [27] found that QBP1 reduces the frequency of mechanostable expanded polyQ protein monomers, presumably by preventing the β -sheet conformational transition (Table 2). To date, QBP1 is the only molecule that has been shown to selectively bind to the expanded polyQ stretch and inhibit its toxic β -sheet conformational transition.

We also tested whether QBP1 can inhibit the subsequent aggregation of the expanded polyQ protein using an *in vitro* assay that we designed previously [25]. In this assay, polyQ stretches fused with thioredoxin (thio-Qn) form aggregates *in vitro* in a time-, concentration-, and polyQ-length dependent manner (for polyQ >40), which mimics the expanded polyQ protein *in vivo*. We found that co-incubation of QBP1 with thio-Q62 dramatically inhibits thio-Q62 aggregation in a concentration-dependent manner, showing an almost complete inhibition at a stoichiometry of 3:1 (thio-Q62:QBP1) [25] (Table 2). The control peptide SCR had no effect on thio-Q62 aggregation. Furthermore, addition of QBP1 after the initiation of thio-Q62 aggregation inhibited any further aggregation, but could not solubilize the aggregates that were already formed, confirming that QBP1 inhibits the earlier stages of the polyQ aggregation process [28]. We also found that (QBP1)₂, a tandem repeat of QBP1 (see Table 1) with a higher binding affinity to the expanded polyQ stretch (K_d 0.6 μ M), presumably due to the additional polyQ binding sequence, exhibits stronger polyQ aggregation inhibitory activity [26]. These results demonstrate that aggregation inhibitory activity is determined by the binding affinity to the polyQ stretch.

Therapeutic Effects of QBP1 on Cell Culture Models

We also examined whether QBP1 could exert therapeutic effects on cell culture models of the polyQ diseases [25]. We co-expressed QBP1 fused with cyan fluorescent protein (QBP1–CFP) together with an expanded polyQ stretch fused with yellow fluorescent protein (polyQ–YFP) in COS-7 cells, which resulted in colocalization of QBP1–CFP with polyQ–YFP inclusion bodies. Importantly, we found that QBP1–CFP inhibited polyQ–YFP-mediated cytotoxicity, as well as inclusion body formation, and its effect was stronger for shorter length polyQ stretches (Table 2). Furthermore, expression of (QBP1)₂–CFP had a more robust therapeutic effect than the single QBP1, which is in accordance with our *in vitro* results [26].

We also investigated the effect of QBP1 on oligomer formation of the expanded polyQ protein in cells, which

Table 1 Polyglutamine binding peptides (QBPs) identified to bind preferentially to the expanded polyQ stretch

Name	Q62/Q19 binding ratio	Sequence (X ₅ -fixed-X ₅)
QBP1	1.66	SNWKWWPGIFD
QBP2	1.31	HWWRSWYSDSV
QBP3	1.30	HEWHWWHQEAA
QBP4	1.27	WGLEHFAGNKR
QBP5	1.25	WWRANWATPVD
QBP6	1.23	WHNYFHWQDQT
(QBP1) ₂	ND	SNWKWWPGIFDSNWKWWPGIFD

ND = not determined.

occurs before inclusion body formation, using fluorescence correlation spectroscopy and fluorescence resonance energy transfer. QBP1 decreased the amount of expanded polyQ protein oligomers, and the effect was greater for shorter length polyQ stretches [29, 30] (Table 2). These results are consistent with our *in vitro* data, which show that QBP1 inhibits the monomeric conformational transition of the polyQ protein that occurs before oligomer and aggregate formation.

Therapeutic Effects of QBP1 by Crossing of Transgenic Animals

As the next step towards developing QBP1 as a therapeutic molecule, we investigated whether overexpression of QBP1 can exert therapeutic effects *in vivo*. For these analyses we used *Drosophila* models of the polyQ diseases, as *Drosophila* are advantageous for various reasons, including their low cost, short lifespan, and ease of genetic manipulation [31]. Transgenic flies expressing an expanded polyQ protein under the control of an eye-specific promoter demonstrate degeneration of the eyes, as well as inclusion body formation, while flies expressing an expanded polyQ protein under a panneuronal promoter exhibit shortened lifespan due to neurodegeneration. Crossing of flies expressing (QBP1)₂-CFP and flies expressing the expanded polyQ protein in the eyes resulted in significant inhibition of eye degeneration, as well as inclusion body formation [32] (Table 2). Co-expression of (SCR)₂-CFP did not affect the

polyQ-induced phenotype at all. Furthermore, crossing of (QBP1)₂-CFP-expressing flies with flies expressing the expanded polyQ protein in neurons resulted in a dramatic extension of lifespan. These results demonstrate clearly the therapeutic effect of QBP1 on polyQ-induced neurodegeneration *in vivo*.

Therapeutic Effects of QBP1 by Exogenous Delivery

Although our results demonstrate the effectiveness of QBP1 *in vivo*, to establish a therapy for the polyQ diseases using QBP1, QBP1 needs to be delivered to vulnerable neurons in the brain, rather than be expressed by the crossing of genetically-engineered animals. For this purpose we have attempted two methods: 1) delivery of the QBP1 transgene using viral vectors and 2) delivery of the QBP1 peptide using protein transduction domains (PTDs).

To deliver the QBP1 transgene into the brains of polyQ disease mice, we used adeno-associated virus vector (AAV) because of its widespread infection throughout the brain, its long-term expression of transgenes, and its safety [33]. Injection of AAV5-QBP1 into the brains of a polyQ disease mouse model resulted in significant inhibition of inclusion body formation (Table 2). However, we were unable to detect the therapeutic effect of AAV5-QBP1 on the neurologic phenotypes of these mice, such as motor impairment and shortened lifespan [34]. As the infection efficiency of AAV5-QBP1 was quite low (~30 %), the extent of neurons expressing QBP1 in the polyQ disease mouse brains was

Table 2 Summary of the effect of polyglutamine binding peptide 1 on polyglutamine (polyQ) disease models

Model/experiment	Phenotype	PolyQ protein	Ref.
<i>In vitro</i>			
Co-incubation	–	Toxic β -sheet transition ↓ Aggregation ↓ Mechanostable monomers ↓	[12] [25] [27]
Cell culture			
Overexpression	Cytotoxicity ↓	Inclusion bodies ↓ Oligomers ↓	[25] [29, 30]
PTD-mediated delivery	Cytotoxicity ↓	Inclusion bodies ↓	[38]
<i>Drosophila</i>			
Overexpression by genetic crossing	Lifespan ↑ Eye degeneration ↓	Inclusion bodies ↓	[32]
PTD-mediated delivery	Lifespan ↑	Inclusion bodies ↓	[38]
Mouse			
AAV5-mediated delivery	No effect detected	Inclusion bodies ↓	[34]
AAV2-mediated delivery	Lifespan ↑ Clasping ↓	Inclusion bodies ↓ Aggregation ↓	[35]
PTD-mediated delivery	Body weight ↑	No effect detected	[39]

PTD = protein transduction domains; AAV = adeno-associated virus vector.

probably insufficient to exert a detectable effect on the phenotypes. In fact, Bauer et al. [35] demonstrated that AAV2-mediated delivery of QBP1 ameliorates the neurologic phenotypes of polyQ disease mice (Table 2). Towards developing viral vector-mediated delivery of the QBP1 transgene as a therapy, several issues, such as control of gene expression and delivery of the virus throughout the patient brain, are anticipated to be solved. Using a blood–brain barrier (BBB)-permeable virus vector, such as AAV9 [36], may help to overcome the brain delivery issue.

For administration of the QBP1 peptide, we utilized PTDs to enable the intracellular delivery of QBP1, which are peptide sequences that are capable of penetrating the cell membrane and entering cells, and can thereby deliver covalently-bound cargo molecules into cells [37, 38]. We found that PTD–QBP1 added to the culture medium was transduced efficiently into cultured cells, and inhibited polyQ-mediated cytotoxicity and inclusion body formation [39] (Table 2). Furthermore, administration of PTD–QBP1 to a *Drosophila* polyQ disease model by addition of the peptide into their food resulted in extension of lifespan and inhibition of inclusion body formation [39], indicating the effectiveness of this method *in vivo* (Table 2). We further tested our strategy in a mouse model of the polyQ diseases [40]. Intraperitoneal injections of PTD–QBP1 slightly ameliorated the body weight loss of polyQ disease mice, indicating a therapeutic effect (Table 2). Unfortunately, we were unable to detect any improvement in the motor phenotypes or any inhibition of neuronal inclusion body formation, probably because PTD–QBP1 does not efficiently cross the BBB in mice. Nevertheless, our studies indicate the potential of PTDs for the delivery of QBP1 to patient brains, and improvement of the BBB permeability of PTD–QBP1 should lead to its development as a potential therapy for the polyQ diseases. For this purpose, we are currently screening for PTDs with high BBB permeability, which will, hopefully, enhance the brain delivery of QBP1.

Another approach we are currently taking is to design low molecular weight chemical analogues of QBP1 with high BBB permeability. Towards this goal we have identified the minimal active sequence of QBP1 (WKWWPGIF) [28], as well as its pharmacophores [41, 42]. We are currently working towards elucidating the structure of QBP1 when bound to the expanded polyQ stretch, which should further aid in the design of small chemical analogues of QBP1.

Effects of QBP1 on Other Neurodegenerative Disease-causing Proteins

As mentioned in the *Introduction*, β -sheet-rich amyloid aggregates are formed not only in the polyQ diseases, but also in many other neurodegenerative diseases, such as AD,

PD, ALS, and the prion diseases. As QBP1 is thought to recognize the β -sheet structure of the expanded polyQ stretch, it may also recognize the toxic conformation of other neurodegenerative disease-causing proteins. Accordingly, Carrion-Vazquez and colleagues [27] tested the effect of QBP1 on other neurotoxic proteins, including mutant A β involved in AD and mutant α -synuclein involved in PD. Interestingly, they found that QBP1 was able to inhibit the aggregation and formation of highly mechanostable monomers of mutant α -synuclein, but not mutant A β . These results highlight the potential of QBP1 as a general therapeutic molecule for a wide range of neurodegenerative diseases, although QBP1 does not simply recognize any β -sheet structure, but appears to have sequence specificity. Indeed, it has been reported that an oligomer specific antibody can inhibit the toxicity of several neurotoxic proteins, including A β , α -synuclein, and polyQ [43], and (-)-epigallocatechin gallate can also inhibit the fibrillogenesis of these proteins [44, 45]. Although the mechanism remains unclear, these molecules, including QBP1, may recognize a unique conformation that is present in many neurotoxic proteins. In addition, QBP1 may not bind to normal proteins with β -sheet structures, as QBP1 has sequence specificity and β -sheets tend to be located within the core of proteins. It will be interesting to see in the future whether QBP1 can exert therapeutic effects against *in vivo* models of diseases, such as PD.

Concluding Remarks

Here we have reviewed our therapeutic strategy against the polyQ diseases using QBP1—a peptide that binds selectively to the expanded, but not normal, length polyQ stretch, probably by recognizing its unique conformation (Fig. 1). We have provided a large body of evidence on the potential of QBP1 as a therapeutic molecule, from its effect on polyQ misfolding/aggregation *in vitro* to its effect on polyQ-mediated neurodegeneration *in vivo* (Table 2).

Although our work has been focused on the polyQ diseases, our approach can also be applied to a broad range of neurodegenerative diseases caused by protein misfolding/aggregation, such as AD, PD, ALS, and the prion diseases. Indeed, various peptides/proteins that inhibit protein aggregation have been reported to exert therapeutic effects in cell culture and animal models of these diseases [46, 47]. Furthermore, the recent study by Carrion-Vazquez and colleagues [27] suggests that QBP1 may, in fact, have potential as a therapy also for at least some of these other protein misfolding diseases, such as PD. We hope that in the near future aggregation inhibitor-based drugs will be developed and bring relief to patients suffering from these currently intractable neurodegenerative diseases.

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