Molecular misreading: the occurrence of frameshift proteins in different diseases[1]

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Abstract

Neuronal homoeostasis requires a constant balance between biosynthetic and catabolic processes. Eukaryotic cells primarily use two distinct mechanisms for degradation: the proteasome and autophagy of aggregates by the lysosomes. We focused on the UPS (ubiquitin–proteasome system). As a result of molecular misreading, misframed UBB (ubiquitin B) (UBB^{+1}) is generated. UBB^{+1} accumulates in the neuritic plaques and neurofibrillary tangles in all patients with AD (Alzheimer's disease) and in the neuronal and glial hallmarks of other tauopathies and in polyglutamine diseases such as Huntington's disease. UBB^{+1} is not present in synucleinopathies such as Parkinson's disease. We showed that UBB^{+1} causes UPS dysfunction, aggregation and apoptotic cell death. UBB^{+1} is also present in non-neurological cells, hepatocytes of the diseased liver and in muscles during inclusion body myositis. Other frequently occurring (age-related) diseases such as Type 2 (non-insulin-dependent) diabetes mellitus are currently under investigation. These findings point to the importance of the UPS in diseases and open new avenues for target identification of the main players of the UPS. Treatment of these diseases with tools (e.g. viral RNA interference constructs) to intervene with specific targets is the next step.

Introduction

The discovery of molecular misreading

Molecular misreading of genes (i.e. the inaccurate conversion of genomic information into aberrant proteins) was demonstrated in the VP (vasopressin) gene of magnocellular neurons in the hypothalamus of the homozygous Brattleboro mutant rat [1]. The mutant trait (diabetes insipidus) is inherited in an autosomal recessive way according to classic Mendelian genetics. Gene cloning revealed the mutation: a single guanine residue deletion in exon B results in a VP precursor protein with a very sticky polylysine C-terminus which is arrested in the endoplasmic reticulum. Consequently, VP is missing from the posterior pituitary of these animals [2]. However, surprisingly, solitary neurons were intensely stained with antibodies against the normal C-terminus of the VP precursor. These neurons, with a revertant VP phenotype, were shown to increase in number with advancing age due to the diseased state of these animals. Analysis of the mRNA of these cells revealed dinucleotide deletions (ΔGA) located downstream of the glycine deletion, in GAGAG motifs. Consequently, at these points, the normal reading frame was restored [3,4].

Subsequently, the question was raised whether this mutation is a peculiarity of the Brattleboro rat or a more general phenomenon. The wild-type VP genes of rat and human have the same GAGAG motifs. Indeed, antibodies directed against the predicted VP sequences in the +1 reading frame resulted in an intense staining of a number of magnocellular neurons [4]. Thus a similar dinucleotide deletion (ΔGA) is likely to occur in wild-type sequences and is not restricted to homozygous Brattleboro rats. It is important to realize that, in these cases, an abnormal VP precursor protein is created out of a normal one. These abnormal +1 proteins are potentially functionally disturbed [1,3]. Molecular misreading also happens in non-neuronal cells, as was shown in transgenic VP mouse lines having ectopic expression of VP [5].

Which transcripts have GAGAG motifs?

The next step was to see if other genes have GAGAG motifs or other simple repeats. The chance of encountering a GAGAG motif is 1:1024, and there are many genomic sequences that have such a potentially error-prone site. In the first instance, we focused on AD (Alzheimer's disease)-associated genes such as APP (amyloid precursor protein) and UBB (ubiquitin B), of which indeed the respective +1 proteins were found as the hallmarks of all cases of AD (including sporadic ones) and Down's syndrome [6]. The proposed dinucleotide deletions were found as well. The existence of these dinucleotide deletions was confirmed independently [7]. Several +1 proteins appeared to co-exist [e.g. UBB^{+1}, $GFAP^{+1}$ (glial fibrillary acidic protein) and APP^{+1}; Figure 1A–1C respectively] due to molecular misreading

Key words: diabetes mellitus (Type 2), frameshift protein, glial fibrillary acidic protein, molecular misreading, polyglutamine disease, ubiquitin.

Abbreviations used: AD, Alzheimer's disease; APP, amyloid precursor protein; CSF, cerebrospinal fluid; GFAP, glial fibrillary acidic protein; HSP, heat-shock protein; UBB, ubiquitin B; UFD, ubiquitin-fusion degradation; UPS, ubiquitin–proteasome system; VP, vasopressin.

[1]**This paper is dedicated to the memory of Cecile Pickart.**

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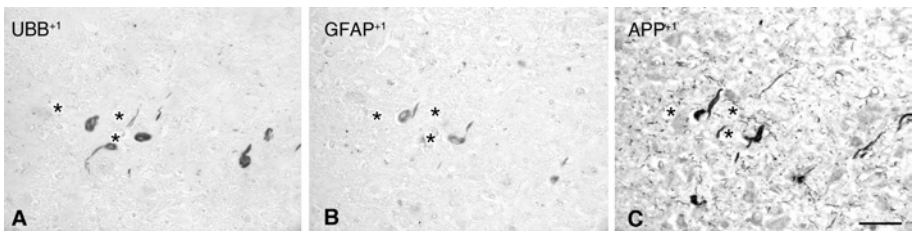
Figure 1 | **Co-existence of various +1 proteins**

Consecutive 6 μm thick paraffin sections of the hippocampus of a 70-year-old-patient suffering from AD showing co-existence of UBB^{+1}, GFAP^{+1} and APP^{+1} in a hallmark of AD, neurofibrillary tangles. Star symbols indicate capillaries for orientation. Scale bar, 25 μm.



(APP and UBB) and exon skipping [8]. We subsequently focused on UBB because of the potentially devastating effects of both a gain or loss of function of the resulting +1 protein in different cellular processes.

UBB and its mutant form: UBB^{+1}

Ubiquitin is a multigene family (i.e. ubiquitins A, B and C), with two polyubiquitin genes encoding multiple repeats of ubiquitin [9,10]. The human UBB gene is localized on chromosome 17p11.2. UBB is one of the best-conserved eukaryotic proteins. From yeast to human, only at three positions does the amino acid sequence show variation in the 76-residue-long protein, indicating its functional relevance. UBB is synthesized in a three repeat from which 76 amino acids are cleaved. The UBB molecule harbours a number of lysine moieties, of which numbers 29 and 48 are involved in ATP-dependent polyubiquitination, a process that triggers transport to the proteasome, followed by proteolysis. Ubiquitin is not only implicated in protein degradation during disease [11,12] but also in an array of other functions (e.g. in synaptic function and plasticity) [13].

At the C-terminus of UBB, a glycine moiety (no. 76) is present, which is essential for all biological functions of UBB. A few nucleotides upstream of this C-terminal glycine moiety, a GAGAG motif is present (Figure 2). The result of a dinucleotide deletion in this motif is a loss of the C-terminal glycine moiety and a 19-amino-acids-longer ubiquitin molecule called ubiquitin^{+1} (UBB^{+1}). It was shown that UBB^{+1} is unable to ubiquitinate, is a UFD (ubiquitin-fusion degradation) substrate for proteasomal degradation and inhibits proteasomal degradation [14] in a dose-dependent way: low levels of UBB^{+1} are degraded, whereas higher levels inhibit the proteasome. In other words UBB^{+1} by itself is degraded but if another cellular stressor (e.g. amyloid β-peptide) is present, UBB^{+1} may become toxic. It was shown that ubiquitinated-UBB^{+1} chains are very refractory to disassembly by de-ubiquitination and potentially inhibit proteasomal degradation by purified 26 S proteasomes [15]. High expression of UBB^{+1} proteins in neuroblastoma cells induces HSP (heat-shock protein) expression (HSP40 and HSP70), resistance to oxidative stress and apoptosis [16,17]. *In vitro*, it was shown that UBB^{+1} enhances aggregation of polyglutamine proteins and synergistically aggravates polyglutamine-induced cell death [18]. The ubiquitin-conjugating enzyme [E2-25K/Hip2 (Huntingtin-interacting protein 2)] has been functionally implicated in the mediation of amyloid β-peptide neurotoxicity and proteasome inhibition by UBB^{+1} *in vitro* [19]. Furthermore, the presence of UBB^{+1} in histological sections is a marker for proteasome impairment in a disease-specific manner in neuronal and glial cells (tauopathies and polyglutamine diseases but not in synucleinopathies) [20], and in non-neuronal cells (Figures 3A–3C) [21,22].

In order to assess if UBB^{+1} protein is functionally relevant to neuronal functioning, we have generated transgenic mouse lines expressing UBB^{+1} protein at low and high levels in the brain (with the Ca^{2+}/calmodulin-dependent protein kinase IIα promoter). We noted UBB^{+1} accumulation in forebrain regions (e.g. the hippocampus and cerebral cortex) in a high UBB^{+1} expression line and a deficit in spatial memory, indicating that optimal activity of the UPS (ubiquitin–proteasome system) is required for cognitive function (D.F. Fischer, P. van Tijn, B. Hobo and F.W. van Leeuwen, unpublished work). Proteomic analyses of the cerebral cortex of these mice taught us differences in the expression pattern of various compounds (e.g. those involved in energy metabolism and in axonal transport) [22a–22c].

Other +1 proteins in diseases

APP^{+1}

APP^{+1} was present in the cellular hallmarks from all tauopathies but not in synucleinopathies [6,23]. APP^{+1} was found to be a secretory protein. Unexpectedly, we found that the APP^{+1} level in the CSF (cerebrospinal fluid) of non-demented controls was much higher than that in the CSF of patients suffering from AD. Also the level of APP^{+1} in CSF was inversely correlated with the severity of neuropathology, especially between Braak stages 0 and 1 [24]. These results were confirmed in the transgenic APP^{+1} mouse [25]. If accumulated, APP^{+1} can increase the levels of secreted Aβ40 (amyloid β-peptide 40) and contribute to AD pathology [26]. In conclusion, measuring levels of APP^{+1} in CSF can help in diagnosing AD at an early stage.

GFAP^{+1}

Overexpression of genes in the course of a disease has been correlated to molecular misreading [1,6]. Along this line, we reasoned that astrocyte proliferation and possibly

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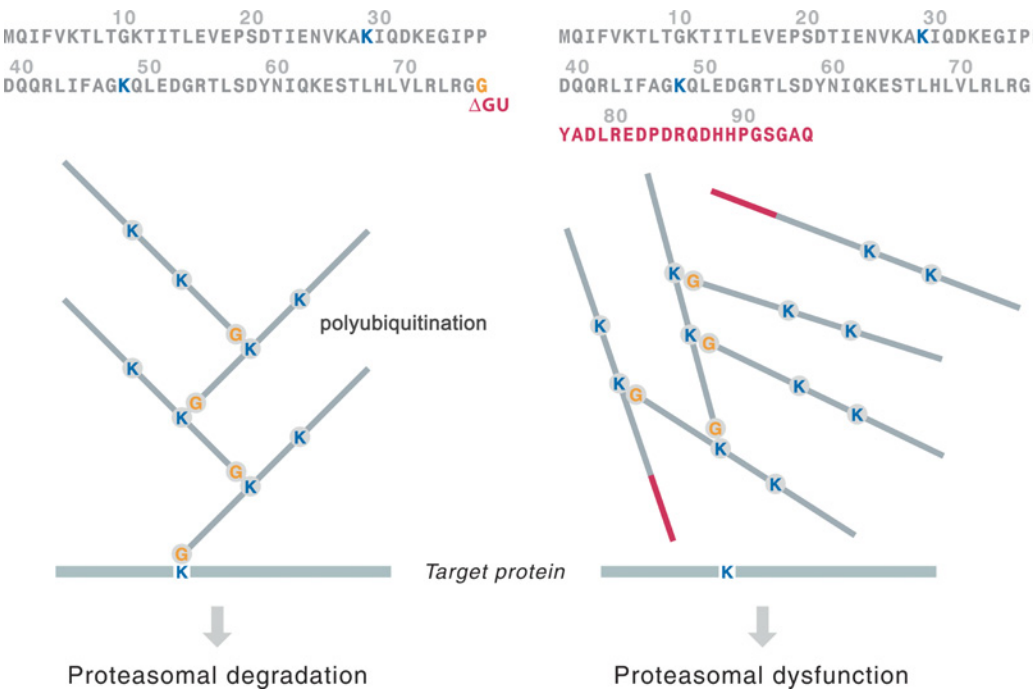
Figure 2 | Polyubiquitination of proteasome substrates

Simplified and schematic representation of how ubiquitin acts via polyubiquitination through a number of enzymatic steps (E_1, E_2 and E_3) (left panel; for details, see [39]). The lysine moieties (K) at positions 29 and 48 are involved in the polyubiquitination and degradation. At the C-terminus of UBB, a GAGAG motif is present. It was shown that adjacent to this motif a dinucleotide deletion (ΔGU) occurs, resulting in an extension of 20 amino acids (right panel). Due to the dinucleotide deletion, the Gly^{76} moiety at the C-terminus, essential for binding to a target protein, is not synthesized. Consequently, this molecule cannot ubiquitinate. In fact, UBB^{+1} is the first naturally occurring UFD substrate identified [14]. Of much interest is that it was recently suggested that E3 enzymes form a 'forked' polyubiquitin chain in which two ubiquitin chains are linked to adjacent lysine residues on a preceding ubiquitin moiety (e.g. Lys^{29} and Lys^{48}). The forked polyubiquitin chains appeared to be relatively resistant to disassembly or degradation by the 26 S proteasomes (A.L. Goldberg and H.-T. Kim, personal communication). It is thus possible that ubiquitinated UBB^{+1} is an inhibitor of the proteasome [15].



GFAP overexpression in AD may result in frameshift GFAP ($GFAP^{+1}$) in reactive astrocytes in AD. To our surprise, we found $GFAP^{+1}$ to be present predominantly in neurons (Figure 3D), suggesting a retrodifferentiation of these neurons [8]. Subsequent analysis of GFAP transcripts revealed that $GFAP^{+1}$ immunoreactivity is due to novel GFAP splice forms [8].

Proteasomal quality control mechanisms may be impaired in neuroepithelial tumours of which the astrocytomas, including glioblastomas, are the most frequent form (80%) [27,28]. However, we did not find any UBB^{+1} immunoreactivity in these tumours. Low-grade astrocytomas are usually associated with high GFAP expression. Indeed $GFAP^{+1}$ protein expression was found in some forms of astrocytomas (in 12 out of 36 cases) but not in oligodendroglioma ($n = 6$) or meningioma ($n = 2$), indicating a need to further analyse these findings and to reveal a possible functional relevance for $GFAP^{+1}$ (Figure 3E) [29].

$p53^{+1}$

Another interesting molecule is p53, known as the tumour-suppressor gene. It contains two GAGAG motifs in exons 8 and 10 [30]. In the first one, present in the DNA-binding region at amino acid residue 280, a dinucleotide deletion (ΔGA) results in $p53^{+1}$ with a novel C-terminus of 24 amino acids. $p53^{+1}$ lacks the oligodimerization domain and nuclear localization and export signals. Against the last 14 amino acids (QERGASPRAAPREH) of $p53^{+1}$, an antibody was raised that resulted in an intense staining of the pyramidal cells of the human hippocampus (F.W. van Leeuwen, unpublished work). However, in neuroepithelial tumours tested so far, no staining was obtained. The GAGAG motif in exon 10 (oligodimerization domain) resulted in an almost immediate stop and was not considered.

Conclusions and future plans

(i) Frameshift proteins may contribute to cellular dysfunction in many diseases. UBB^{+1} has dual substrate/inhibitor effects on the proteasome. If UBB^{+1} accumulates, it causes proteasomal dysfunction in various neuronal and non-neuronal diseases.

(ii) Many forms of (age-related) diseases are sporadic and multifactorial. This means that in the next years the generation of more multiple transgenic animals such as those for AD may

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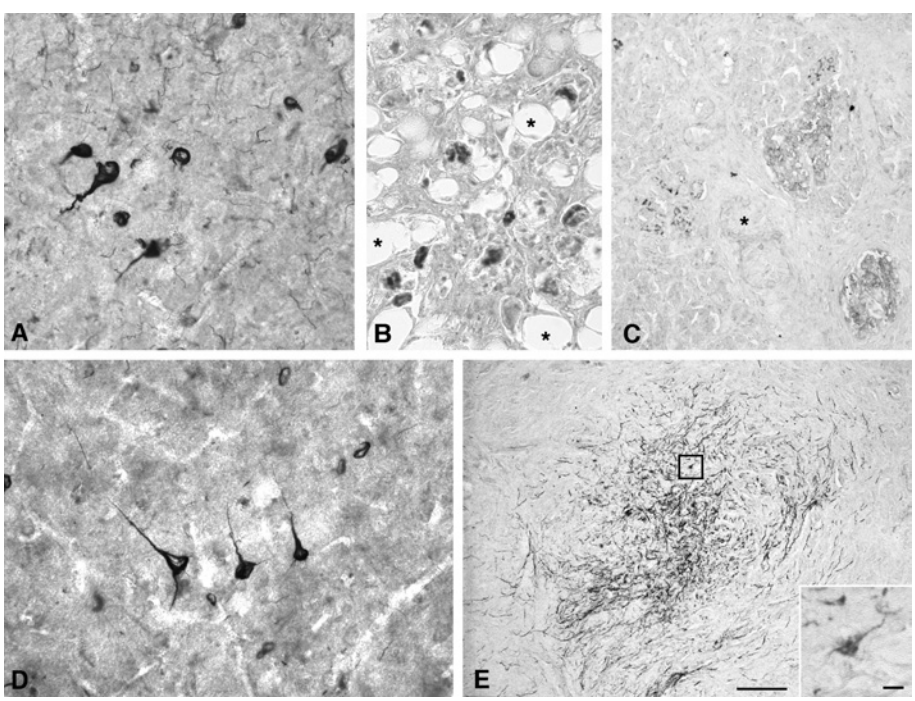
Figure 3 | UBB^{+1} and GFAP^{+1} in various diseases

UBB^{+1} (**A**–**C**) and GFAP^{+1} (**D**, **E**) staining in 50 μm Vibratome (**A**, **D**) and 6 μm thick paraffin sections (**B**, **C**, **E**). In (**A**) and (**C**) in the hippocampus of a 92-year-old patient suffering from AD, intense immunoreactivity can be seen in neurofibrillary tangles. Disease-specific expression of UBB^{+1} has been found in tauopathies. However, in FTD (frontotemporal dementia), UBB^{+1} is only present in three of eight examples, reflecting the heterogeneity in the clinical and pathological phenotypes [20]. In synucleinopathies [LBD (Lewy body disease) and MSA (multiple system atrophy)], UBB^{+1} is absent [20]. Remarkably, GFAP^{+1} accumulation was only detected in AD and in two of eight cases of Pick's disease. In other tauopathies (progressive supranuclear palsy, $n = 5$; FTD, $n = 3$; and argyrophylic grain disease, $n = 5$), in synucleinopathies (Parkinson's disease/LBD, $n = 20$; and MSA, $n = 5$) and in multiple sclerosis ($n = 11$), no GFAP^{+1} immunoreactivity was observed. In (**B**), UBB^{+1} staining is present in Mallory bodies of the liver of a 53-year-old patient with alcohol abuse and liver cirrhosis (no. 7) [20]. Star symbols in this Figure indicate lipid droplets. No staining was obtained with anti-APP^{+1} and anti-GFAP^{+1} antibodies in nine patients with alcoholic liver disease and Mallory bodies [21]. In (**C**), UBB^{+1} staining is shown in the endocrine pancreas using three different anti-UBB^{+1} antibodies [20]; heterogeneous staining of β-cells in the islets of Langerhans of a person aged 65 years with a history of hypertension (not a diabetes mellitus patient). Note also non-reactive islets located in between (star symbol). In (**E**), GFAP^{+1} staining is present in cell bodies (inset) of astrocytes and fibres in the brainstem of a female (aged 12 years) with a pilocytic astrocytoma type 1. With APP^{+1} and UBB^{+1} antisera, no staining was obtained. Scale bars, 25 μm (**E**) and 10 μm (inset).



be expected (see e.g. [31]). In these models, alterations in the UPS genes may be considered as relevant contributors to the disease phenotypes.

(iii) The presence of UBB^{+1} in affected neurons in AD, other tauopathies and several polyglutamine diseases, but not in synucleinopathies (Figures 1A and 3A), may be used as a marker for proteasome insufficiency. However, it has been reported that the proteasome is not only inhibited in AD [32] and Huntington's disease (sharing UBB^{+1} accumulation) but also in Parkinson's disease [33]. We surmise that the mechanisms leading to proteasomal insufficiency are different in these diseases. Whereas in Parkinson's disease the ubiquitination and de-ubiquitination machinery may falter, in AD UBB^{+1} inhibition of the proteasome may act at the cap, potentially due to clogging of 19 S subunit(s) with substrates such as ubiquitinated UBB^{+1} [15,20,34,35]. Indeed, genetic data point to a role for ubiquitination enzymes in familial Parkinson's disease [Parkin and UCH L-1 (ubiquitin C-terminal hydrolase L-1)] [36]. These differences may result in specific UPS targets that can be corrected by viral tools. RNA silencing techniques to neutralize UPS transcripts or viral technology to compensate for their lack are the candidates for therapy as has been shown in polyglutamine diseases such as Huntington's disease [37,38].

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