Linear and extended: a common polyglutamine conformation recognized by the three antibodies MW1, 1C2 and 3B5H10

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A long-standing pathomechanistic model proposes that the polyglutamine (polyQ)-length-dependent toxicity threshold observed in all polyQ diseases is triggered by a conformational change within the monomer that occurs only above a certain polyQ length. If true, this yet undefined and elusive mutant-specific toxic conformation would constitute a direct therapeutic target. Three anti-polyQ antibodies—MW1, 1C2 and 3B5H10—have been extensively used to probe the conformation of polyQ. The crystal structure of the MW1 epitope reveals a linear, non-pathogenic polyQ. In contrast, although the detailed structure of its epitope is unknown, the 3B5H10 antibody is widely advertised and used as a conformational antibody that recognizes the toxic conformation of expanded polyQ. We solved the crystal structure of the 1C2 antigen-binding domain (1C2-Fab) and performed a direct comparison between the 1C2, MW1 and 3B5H10 structures. The MW1 and 1C2 antibodies have similar sequences and structures, consistent with their binding to short polyQ and their polyQ length-discrimination properties. Unexpectedly, the 3B5H10 antibody also shares striking features with MW1 and 1C2, which prompted us to revisit its binding properties. We show that the 3B5H10 epitope is actually a short, non-pathogenic polyQ. All three antibodies MW1, 1C2 and 3B5H10 interact similarly with polyQ of various lengths, and bind small polyQ epitopes in similar linear and extended conformations. Together with studies published during the recent years, our work argues against the hypothesis that a mutant-specific conformation in monomeric polyQ molecules is the toxic entity responsible for polyQ diseases.

INTRODUCTION

Polyglutamine (polyQ) disorders are a group of nine inherited neurodegenerative disorders caused by the aberrant expansion of a trinucleotide repetition (CAG)n within the coding region of a protein. These pathologies become penetrant above a certain size threshold that varies among polyQ diseases and reflects the gain of toxic functions of mutant polyQ proteins (1). It has been proposed that the polyQ-length-dependent toxicity threshold is triggered by a conformational change within the monomer that occurs only above a certain polyQ length. This stable and aggregation-prone conformation would convey toxic properties to the soluble monomeric mutant protein and provoke aberrant interactions with its partners (2–9). This ‘structural toxic-threshold’ model has been challenged by another model, in which polyQ tracts are inherently toxic sequences whose deleterious effects gradually increase with polyQ length and aggregation kinetics (10–19). In the latter model, polyQ toxicity manifests once the protein homeostasis machinery, which normally prevents proteotoxicity, is overwhelmed by the accumulation of toxic aggregated species of mutant polyQ proteins.

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Addressing whether monomeric expanded polyQ of pathogenic length can adopt a unique and toxic conformation remains an important question as such a ‘mutant conformation’ would constitute a direct therapeutic target. Anti-polyQ antibodies have been commonly used as tools to discriminate different protein conformations. In particular, three antibodies share the ability to interact specifically and strongly with monomeric expanded polyQ and are extensively used in the polyQ research field: the 1C2, MW1 and 3B5H10 antibodies (3,9,20). We initially proposed that the strong interaction of 1C2 with mutant polyQ could reflect the existence of a mutant polyQ conformational epitope (9). However, we and others later demonstrated that the strong interaction of 1C2 and MW1 is not due to the recognition of a conformational epitope, but due to avidity—a phenomenon in which the valence of both the antibody and the antigen can result in enhanced binding capacity (10,14). The crystal structure of MW1 bound to a non-pathogenic Q10 peptide further revealed that the polyQ epitope adopts a linear and extended conformation (21). More recently, the X-ray crystal structure of unbound 3B5H10 was reported (22), and although the X-ray structure of 3B5H10:polyQ complex has not been determined, it is proposed—based on cell-imaging, biochemical, small-angle X-ray scattering and modelling—that the 3B5H10 antibody specifically recognizes a monomeric and toxic mutant polyQ conformation that triggers neuronal death (20,22). If true, this antibody, which is widely advertised and used in the polyQ field as a conformational antibody, would be a unique tool that could help in understanding the structure and toxicity relationship of mutant polyQ.

Here, we solved the crystal structure of the antigen-binding domain (Fab) of the 1C2 antibody and compared the primary sequences and 3D structures of 1C2, MW1 (apo and holo) and 3B5H10, in order to better understand the structural determinants that would allow different antibodies to tell apart various polyQ conformations. Surprisingly, we found that both the sequences and 3D structures of 3B5H10, 1C2 and MW1 are strikingly similar. Furthermore, we demonstrate that 3B5H10, like 1C2 and MW1, binds polyQ as short as Q11 with micromolar affinities. We conclude that all three antibodies recognize non-pathogenic polyQ in a linear and extended conformation.

RESULTS

Crystal structure determination of 1C2-Fab

We solved the X-ray structure of 1C2-Fab apo form in two different space groups (Supplementary Material, Table S1 and Fig. S1). In addition, to determine the conformation of the polyQ epitope recognized by the antibody, we aimed at crystallizing 1C2-Fab holo form in complex with a polyQ peptide. The length of the polyQ epitope of 1C2-Fab was previously determined to be around Q11 (14). Accordingly, the purification of 1C2-Fab involves an affinity step with Q11 (Supplementary Material, Fig. S1a). We thus attempted to co-crystallize 1C2-Fab with polyQ peptides ≤ Q11 using several strategies (Supplementary Material, Fig. S2a). Although stable complexes between 1C2-Fab and Q10 peptides form in solution (Supplementary Material, Fig. S2a), we failed to obtain co-crystals of 1C2-Fab:polyQ complex. This is likely due to the Fab crystal packing blocking its antigen-binding surface (Supplementary Material, Fig. S2b), which results in the crystallization of the 1C2-Fab apo form only. To circumvent this limitation, we attempted to co-crystallize the minimal domain of interaction of 1C2 (1C2-Fv) with polyQ peptides (Supplementary Material, Fig. S2c), a strategy that was successful to obtain the MW1:Q10 structure (21). Although 1C2-Fv actively bound polyQ in solution, as determined by biochemical and surface plasmon resonance (SPR) (Supplementary Material, Fig. S2d and e), we did not obtain 1C2-Fv crystals, neither in apo nor in holo forms.

Taking advantage of the apo 1C2-Fab and the recently published apo 3B5H10 crystal structures, we thus performed a thorough comparison of the three antibodies MW1, 1C2 and 3B5H10 in search of variations in their antigen-binding surfaces, which would explain their binding different polyQ conformations.

Comparison of the light chain variable domains (Lv) of 1C2, MW1 and 3B5H10

We compared the primary sequences and 3D structures of the Lv domains of 1C2, MW1 and 3B5H10. All three antibodies contain a Lv domain encoded by a rare variable Vx light chain gene present in less than 1% of normal mice antibodies (23,24) and known to undergo very little somatic mutations (25). Consequently, the Lvx amino acid sequences of 1C2, MW1 and 3B5H10 are almost identical (Fig. 1A). We searched in the Protein Data Bank (PDB) for other antibodies carrying an Lv domain encoded by this rare Vx gene, which could help us to understand how 1C2, MW1 and 3B5H10 interact with their epitopes. We found only two other Vx-encoded antibodies of known 3D structure: 13F6-1-2, an antibody that recognizes an Ebola virus glycoprotein [26, PDB code 2QHR] and a monoclonal antibody directed against the parathyroid hormone-related protein: anti-PTHRP [27, PDB code 3FFD]. We also included in our comparisons the F28C4 antibody directed against an encephalitogenic peptide because, although its 3D structure remains unsolved, detailed binding properties and epitope conformation are available and rendered them relevant to our analysis (28,29).

As expected, the Lvx sequences of these antibodies are also highly homologous to the Lvx chains of 1C2, MW1 and 3B5H10 (Fig. 1A). Consequently, the 3D structures of the Lvx domains of 1C2, MW1, 3B5H10, 13F6-1–2 and anti-PTHRP are extremely similar (Fig. 1B). The three complementarity-determining region (CDR) loops L1, L2 and L3 of Lvx are longer than in other Lv subtypes and their conformations may represent new canonical classes for Vx-encoded light chains, as proposed by Lee et al. (26).

Indeed, comparison of apo (1C2, MW1 and 3B5H10) and holo (MW1, 13F6-1–2 and anti-PTHRP) structures reveal only minute variations in the conformations of loops L1 and L2, which are rigid and unaffected by ligand binding (Fig. 1B–D). The loop L3 is more flexible, as illustrated by its important conformational changes observed between apo and holo structures of MW1 [(21) and Fig. 1B], or between two neighbouring apo MW1-Fv molecules in the same crystal (Supplementary Material, Fig. S3a). Nevertheless, we found that loop L3 adopts a very similar conformation in the apo structures of 1C2 and 3B5H10, and in one apo MW1 molecule (Fig. 1E). The only variations observed mostly concern solvent-exposed and less structured...
Figure 1. Primary sequence and 3D structure comparison of the MW1, 1C2 and 3B5H10 antibodies. (A) Primary sequence alignments of the L\(\alpha\)x domains of the indicated antibodies: the compared L\(\alpha\)x sequences are highly similar. The upper numbering corresponds to the 1C2 sequence, and the lower numbering to the Kabat scheme. The amino acids that differ from the other sequences are shaded in dark grey. The CDR loops defined as in Lee et al. (26) are shaded in light grey. The amino acids shaded in orange indicate discrepancies between the sequences of 3B5H10 associated with the structures 3S96.pdb and 4DCQ.pdb (22), see also Supplementary Material, Fig. S3b and c. The stars represent the amino acids of MW1 that are in contact with the polyQ peptide in the MW1:Q10 structure (21). The black stars represent two conserved L\(\alpha\)x amino acids (T32 and G95, 1C2 numbering) that bind a glutamine present in different L\(\alpha\)x ligands peptides (21,26,27), see also (F). (B) Superimposition of the L\(\alpha\)x domain structures of MW1 apo (grey = 2GSG.pdb), MW1 holo (green = 2OTU.pdb, cyan = 2OTW.pdb), 1C2 apo (orange = 4ISV.pdb, beige = 4JJ5.pdb), 3B5H10 apo (red = 3S96.pdb, purple = 4DCQ.pdb), 13F6–1–2 holo (dark blue = 2QHR.pdb) and anti-PTHRP holo (light-blue = 3FFD.pdb). (C and D) Superimposition of the L1 (C) and L2 (D) loops of all antibodies presented in (B) [structures and colour codes as in (B)]. (E) Superimposition of the L3 loop observed in the structures of apo 1C2, 3B5H10 and MW1 [same colour codes as in (B)]. (F) In three different ligand:antibody structures, a glutamine residue from the ligand adopts a similar conformation and mediates similar contacts on the surface of the L\(\alpha\)x domain. MW1:Q10 (green = 2OTU.pdb, cyan = 2OTW.pdb), 13F6–1–2: (blue = 2QHR.pdb) and anti-PTHRP (yellow = 3FFD.pdb). The distances in Angstrom (d1; d2; d3) are: MW1:Q10 (2.8; 2.9; 3.0), 13F6–1–2: (2.9; 3.1; 2.9) and anti-PTHRP (2.8; 3.4; 2.9). T32 and G95 (1C2 numbering) are labelled with a black star in A. (G) Primary sequence alignments of the Hv domains of MW1, 1C2 and 3B5H10: the numbering, shading colour and star codes are identical to (A). Amino acids that are unique to each sequence are shaded in black. The additional box within CDR H3 indicates that the main chain of these amino acids adopt a different configuration in all three antibodies, whereas the surrounding amino acids are in a similar configuration in the three structures. (H) Superimposition of Hv domain structures of MW1 (grey = 2GSG.pdb), MW1:Q10 (green = 2OTU.pdb, cyan = 2OTW.pdb), 1C2 (orange = 4ISV.pdb, beige = 4JJ5.pdb) and 3B5H10 (red = 3S96.pdb, purple = 4DCQ.pdb). (I and J) Superimposition of the H1 (I) and H2 (J) loops of 1C2 and 3B5H10 [colour codes as in (H)].
aminos with elevated B-factors (I_{38}-Q_{101}, Fig. 1E and Supplementary Material, Fig. S1c and d).

Given the high homology of sequence and 3D structure of different LVβx antibody domains, common structural determinants may underlie their reported binding properties to their respective ligands (26). Indeed, the interaction of 13F6-1-2, F28C4 and MW1 with their ligand peptide is atypical and differs from most antibody-peptide structures. Usually, the ligand peptide lies at the light chain/heavy chain interface and is mainly in contact with the heavy chain (21,26). In contrast, the ligand peptides of 13F6-1-2, F28C4 and MW1 adopt a linear and extended conformation (21,26,29). Moreover, the ligand peptides of 13F6-1-2 and MW1 lie diagonally in a shallow groove formed at the surface of the light and heavy variable domains, and mediate important contacts with the LVβx domain: 27% of the buried surface area for 13F6-1-2 (26), and 55% of all contacts for MW1 (21). Importantly, all LVβx structures display an almost identical groove at their surface, which strongly suggests that the LVβx domains of 1C2, 3B5H10 and MW1 could bind a polyQ stretch in a similar manner.

Interestingly, the affinity of F28C4 and 13F6-1-2 for their corresponding antigen was shown to be strongly dependent on the presence of a glutamine in the epitope (26,29). Lee et al. (26) proposed that the anchoring of this glutamine on germline LVβx residues might constitute a general binding rule on the shallow groove of an LVβx domain. As shown in Figure 1F, the MW1:polyQ structural interaction reported by Li et al. (21) supports this hypothesis. Similarly, we show that, although the peptide recognized by anti-PTHRP is helical (27), it also contains a glutamine in a conformation that allows contacts with the same conserved residues of the LVβx domain (Fig. 1F). Hence, it is very likely that the interactions of 1C2 and 3B5H10 LVβx domains with polyQ ligand are also mediated by a similar binding rule involving a key glutamine and specific LVβx residues.

All together, the high primary sequence and structural homology of 1C2, 3B5H10 and MW1 LVβx domains, their highly similar shallow groove at the ligand binding site and the specific structural determinants of LVβx:antigen binding revealed by these comparisons suggest that the 1C2 and 3B5H10 LVβx domains should bind a polyQ peptide in a conformation very similar to that observed in the MW1:polyQ crystal structure.

**Comparison of the heavy chain variable domains (Hv) of 1C2, MW1 and 3B5H10**

The Hv sequences of 1C2, MW1 and 3B5H10 are more divergent than the Lβ chains, with their CDR H3 being the most variable part, both in sequence and in length (Fig. 1G). Accordingly, the 3D structures of the Hv domains of 1C2, MW1 and 3B5H10 are more disparate than the Lβ ones, especially in the H3 region (Fig. 1H).

Although the MW1 sequence is globally more distant to that of 1C2 and 3B5H10, some similarities exist between the three antibodies, such as an enrichment of Hv-CDRs in aromatic residues (22), and the fact that H1, responsible alongside H3 for interactions between Hv-MW1 and polyQ (21), interacts with polyQ. Moreover, if CDR H3 is omitted, the Hv sequences of 1C2 and 3B5H10 display a remarkably high homology (94 identical amino acids of 108 = 87% identity), and their CDR H1 and H2 only vary by a single amino acid, respectively (Fig. 1G). The overall 3D structures of Hv-1C2 and Hv-3B5H10 are extremely similar, as underlined by their loops H1 and H2, with the exception of their H3 (Fig. 1H–J). Furthermore, homology searches with the program BLAST (30) identifies 3B5H10 as the second closest homolog of the 1C2-Fab heavy chain out of 2911 hits in the PDB with an E-value of <0.01 (E-value = 2.48237×100). The closest homolog identified was the heavy chain of the anti-cholera toxin TE33-Fab (E-value = 6.75231×102). The light chains of TE33-Fab and 3B5H10, however, lack remarkable homology, and the TE33-Fab recognizes non-polyQ-related antigens (31).

In summary, when taking into account the sequences of both their light and heavy chains, 1C2 and 3B5H10 are the closest homologs found in the PDB. Furthermore, the 3D structures of L1, L2, L3, H1 and H2 CDR loops of 1C2 and 3B5H10 exhibit striking similarities, and only the H3 loop significantly differs in the two antibodies.

**3B5H10, like MW1 and 1C2, interacts with micromolar affinity with small, non-pathologic polyQ**

The strong sequence homology and structural similarities between 3B5H10, 1C2 and MW1 led us to consider the possibility that 3B5H10 could recognize non-pathogenic polyQ, as do 1C2 and MW1. To clarify this point, we analyzed the interaction properties of 3B5H10 with polyQ spanning from non-pathogenic to pathogenic lengths, or flanked with various sequences.

We performed GST pull-downs using well-characterized GST-polyQ proteins (14,15) to study the ability of 3B5H10 to interact with polyQ of various non-pathogenic (Q_{11}, Q_{22}) and pathogenic (Q_{41}) lengths. We found that 3B5H10 can specifically bind to polyQ as small as Q_{11} (Fig. 2A).

The SPR technique was previously used to show that the Fabs of MW1 and 1C2 have a micromolar affinity for polyQ stretches of non-pathogenic and pathogenic lengths, and to conclude that they do not specifically recognize a mutant-specific polyQ conformation (10,14). We thus studied by SPR the binding properties of 3B5H10-Fab with polyQ of various lengths, using 1C2-Fab as a control. As shown in Figure 2B, the intensity of the SPR response at the end of the injection phase (R_{120s}) increased as a function of the polyQ length for all concentrations of 3B5H10-Fab or 1C2-Fab injected on immobilized GST-polyQ proteins. This phenomenon has already been documented for MW1 and 1C2, and can be explained by the progressive valence increase of the polyQ sequence, which allows longer polyQ stretches to interact simultaneously with several Fabs (10,14). A similar effect was observed on western blot (WB) when using the Fabs of 3B5H10 or 1C2 as primary antibodies, as the intensity of the signal increased progressively as a function of polyQ length (Fig. 2C).

Interestingly, soluble polyQ—of non-pathogenic and pathogenic lengths, and surrounded by different flanking sequences—is a disordered homopolymer (10,11,14,15,32,33), bearing a number of epitopes that increases in parallel with polyQ length (e.g. in the case of MW1, it increases from 13 epitopes in Q_{22} to 32 epitopes in Q_{41}, Supplementary Material, Fig. S4a). The multiplicity of epitopes, combined with the possibility of several Fabs interacting simultaneously on longer polyQ, constitute thus an unusually complex interaction system [Fig. 2B and C, Supplementary...
Therefore, conditions can be found to detect only the longer polyQ, even when similar quantities of short and long polyQ are present: for example, the Q41 stretch, but not the Q11 one, is detected with the lower concentrations of 1C2 and 3B5H10-Fab’s (Fig. 2B and C). This was already illustrated in similar WB experiments previously performed by Bennett et al. (10) with MW1 and 1C2, by us with 1C2 (14) and by Miller et al. (20) with 3B5H10, where a Q68 stretch was strongly revealed by the Fab or IgG form, but a smaller Q17 sequence was only weakly detected by the full antibody. Importantly, the polyQ-length-dependent increase in R120s and WB responses (Fig. 2B and C) is progressive and is not specific to non-pathogenic/pathogenic length transitions (Q22 to Q41), since it also occurs between two non-pathogenic lengths (Q11 to Q22).

Figure 2. Interaction of the 3B5H10 and 1C2 antibodies with polyQ of various lengths. (A) GST pull-down assays of 3B5H10 and 1C2 on GST-polyQ bearing different polyQ lengths: 3B5H10 and 1C2 come down in the presence of GST-Q11, GST-Q22, or GST-Q41 but not GST. The Coomassie blue stained gel shown here is representative of three pull-down experiments. In one experiment, the samples were also studied in parallel by WB, using a Goat-Anti-Mouse (GAM) antibody coupled to peroxidase that reveals only the heavy chain of 1C2 and 3B5H10 antibodies [Vx-encoded chains are not recognized by most GAM sera (41)]. The WB signal revealing both antibodies on the GST-polyQ lanes is saturated, whereas almost no signal is detectable on the negative-control GST lane, showing that the interactions of 1C2 and 3B5H10 with GST-Q11, GST-Q22, or GST-Q41 are highly specific. (B) SPR analysis of interaction of 3B5H10-Fab and 1C2-Fab with polyQ of various lengths: the two antibodies display similar binding properties with polyQ of various lengths. GST (negative control and reference), GST-Q11, GST-Q22, and GST-Q41 were linked by an anti-GST antibody [as in Klein et al., (14)] on different channels of the same SPR chip [93, 110, 97 and 90 relative units (RU), respectively]. Different concentrations of 3B5H10-Fab and 1C2-Fab (from 62.5 to 2000 nM) were successively injected on the SPR chip. The SPR sensorgrams show the specific binding signal of increasing concentrations of 1C2-Fab or 3B5H10-Fab on GST-Q11, GST-Q22 or GST-Q41. For a given concentration of Fab, the SPR response at the end of the injection phase (R120s) increases progressively in a polyQ-length-dependent manner. This figure is representative of two independent experiments that included replicates for the 3B5H10-Fab injections. Kinetic and affinity parameters returned from regression analysis of the Fab-Qn curves are reported in Supplementary Material, Figure S4b. (C) Interaction of 3B5H10-Fab and 1C2-Fab with polyQ of various lengths as analyzed by WB: the efficiency of detection of polyQ stretches by WB, employing 1C2-Fab or 3B5H10-Fab as primary antibodies, increases in a polyQ-length-dependent manner. Equal amounts of GST-Q11, GST-Q22 and GST-Q41 were mixed and loaded in a unique well. After SDS–PAGE and transfer onto a nitrocellulose membrane, the membrane was cut into 15 equivalent lanes. Each lane was individually incubated with the following primary antibodies, at the indicated concentrations: 1C2 IgG (lane 1); 1C2-Fab (lanes 2 to 7); anti-GST (lane 8); 3B5H10-IgG (lane 15). The 15 lanes were then reassembled, incubated with GAM-peroxidase and revealed simultaneously for 30 s (top panel) or 1110 s (lower panel). Note the equal amounts of the three GST-polyQ proteins as revealed with anti-GST after a 30 s exposure. (D) Determination of the affinity of 3B5H10 and 1C2 for GST-Q11 and MBP-Htt-exon1-Q16 by SPR: 3B5H10 and 1C2 have similar affinity for GST-Q11 and MBP-Htt-exon1-Q16. GST-Q11, MBP-Htt-exon1-Q16 and GST (negative control and reference) were cross-linked on different channels of the same SPR chip (37, 96 and 45 RU, respectively). The figure shows the specific binding of 3B5H10 and 1C2 that were injected at different concentrations. The pattern of interaction of each antibody with GST-Q11 and MBP-Htt-exon1-Q16 are similar, and both antibodies have a micromolar affinity for GST-Q11 and MBP-Htt-exon1-Q16 (Supplementary Material, Fig. S4c).
We estimated by SPR that the affinities of 3B5H10-Fab or 1C2-Fab for Q_{11}, Q_{22} and Q_{41} sequences are all in the micromolar range, with very little variation from Q_{11} to Q_{41} (Supplementary Material, Fig. S4b), consistently with the previous characterization of 1C2 and MW1 antibodies by SPR (10,14,21). Moreover, to assess the potential influence of sequences flanking the polyQ stretch on the interaction with 3B5H10 and 1C2, we also studied their binding with non-pathogenic polyQ stretches surrounded by different flanking sequences: Q_{11} bound to GST (GST-Q_{11}) and Q_{3} in the context of the Huntingtin exon-1 fused to an MBP carrier [MBP-Htt-exon1-Q_{16}] (12)]. We found that the sequences flanking the polyQ stretch do not influence the antibody:polyQ interaction and that both antibodies have similar micromolar affinities for Q_{11} and for Htt-exon1-Q_{16} (Fig. 2D and Supplementary Material, Fig. S4c).

It is noteworthy that the interaction properties of 1C2 and 3B5H10 with polyQ are not exactly the same (Fig. 2B–D). This is not surprising, since 1C2 and 3B5H10 differ significantly for the H3 CDR domain (Fig. 1), and are thus not identical. However, our results show that the 3B5H10, MW1 and 1C2 interact with polyQ of various lengths in an extremely similar fashion and that the epitope recognized by 3B5H10 is not a mutant polyQ-specific conformation, but can be a non-pathologic polyQ stretch as small as Q_{11}.

**DISCUSSION**

MW1, 1C2 and 3B5H10 recognize a non-pathogenic polyQ in a linear and extended conformation

The three anti-polyQ antibodies MW1, 1C2 and 3B5H10 were generated independently, in three different laboratories and with three different antigen contexts containing polyQ stretches of different lengths: 1C2 was generated against the TATA-Binding Protein carrying Q_{38}, MW1 against the Atrophin-1 carrying Q_{19} and 3B5H10 against an amino-terminal Huntingtin fragment carrying Q_{66} (3,9,20). Despite the dissimilarity of antigens, all three antibodies share very striking similarities: their interaction properties with polyQ stretches, as studied with different techniques, are remarkably alike [(10,14,21) and see Fig. 2 and Supplementary Material, Fig. S4]; they all carry a rare variable light chain L\(\text{\nu}{\alpha}\) domain, which differs from other L\(\text{\nu}{\alpha}\) domains through its important role in binding antigenic peptides in an extended conformation (21,25,26,29); the L\(\text{\nu}{\alpha}\) domains of the three antibodies have almost identical sequence and 3D structure shaping a similar groove at the ligand-binding surface (Fig. 1). In addition, the Hv moieties of 1C2 and 3B5H10 are also very similar (Fig. 1G–J). The remarkable likeness of MW1, 1C2 and 3B5H10 suggests that during the immune response process in the mouse, elicited by different antigenic contexts through its important role in binding antigenic peptides in an extended conformation (21,25,26,29), the L\(\text{\nu}{\alpha}\) domains of the three antibodies have almost identical sequence and 3D structure shaping a similar groove at the ligand-binding surface (Fig. 1). In addition, the Hv moieties of 1C2 and 3B5H10 are also very similar (Fig. 1G–J). The remarkable likeness of MW1, 1C2 and 3B5H10 suggests that during the immune response process in the mouse, elicited by different antigenic contexts, a similar polyQ epitope was used for the generation of the three antibodies.

The unique atomic structure of a polyQ peptide bound to the MW1 antibody shows that the MW1 epitope consists of a small Q_{10} peptide in an extended conformation, sitting non-canonically in a shallow groove on the surface of the L\(\nu\) and Hv domains [(21,26), see Fig. 3A]. The L\(\nu\alpha\) domain of MW1 mediates 55% of all contacts with the Q_{10} peptide, and 6 out of 10 glutamates are sitting on the L\(\nu\alpha\) surface. To bind the Q_{10} peptide, the antigen-binding surface of MW1 undergoes some structural rearrangements, including important conformational changes of the L3 and H3 CDR loops (Fig. 3B). Since the L\(\nu\alpha\) domains of MW1, 1C2 and 3B5H10 display more than 97% identity (Fig. 1A) and their 3D structures reveal an almost identical shallow groove at their ligand-binding interfaces (Fig. 1C–E), these L\(\nu\alpha\) domains should all interact with a polyQ peptide in an almost identical manner. To visualize the binding of polyQ to the L\(\nu\alpha\) moieties of 1C2 and 3B5H10, we positioned a 6-glutamines peptide in an extended conformation on their surface (Fig. 3C and D), as observed for the MW1:Q_{10} structure (Fig. 3A). A structural change similar to that seen for MW1 L\(\nu\alpha\) (Fig. 3B) is likely to occur on the surface of 1C2 and 3B5H10, to fully accommodate the linear and extended Q_{6} stretch. Since 1C2 and 3B5H10 recognize polyQ as short as Q_{10} or Q_{11} [see Fig. 2, Supplementary Material, Fig. S2a and (14)], the four to five remaining glutamines that are not in contact with the L\(\nu\alpha\) domain are likely to be positioned in the groove formed by the hyper-variable loops of the H\(\nu\) domain of 1C2 and 3B5H10, in an unknown conformation (Fig. 3C and D). Given the major contribution of the L\(\nu\alpha\) moiety to the interaction, the polyQ epitopes of 1C2 or 3B5H10 are thus mostly linear and extended.

The existence of a mutant-specific toxic conformation in monomeric polyQ is controversial

A long-standing pathomechanistic model, which we previously referred to as ‘structural toxic-threshold’ model (14), postulates that a mutant-specific and monomeric polyQ conformation is the toxic entity responsible for polyQ diseases. This model was initially proposed based on differences observed between wild-type and mutant polyQ proteins: expanded polyQ was shown to be specifically recognized by the 1C2 antibody (9) and to perturb the interaction of a carrier protein with a partner (5); moreover, only expanded polyQ proteins were initially found to aggregate in vitro and in vivo and to be toxic (8,34). Therefore, it was proposed early on that monomeric polyQ above a certain length threshold can adopt a unique, stable, aggregation-prone and toxic conformation, which can be specifically recognized by antibodies, and which can perturb protein interactions (5,6,8,9).

However, the properties of polyQs were later studied under new experimental conditions, and cumulative evidence now contradict the ‘structural toxic-threshold’ model: in contrast with earlier findings, non-expanded polyQ can form aggregates in vitro, although at much lower kinetics and/or at higher concentrations, and these aggregates resemble those of expanded polyQ (11,14); non-expanded polyQ can also be toxic when administered as aggregates in cellular cultures (19), or when overexpressed in C. elegans (16), showing that toxicity is not an exclusive property of elongated polyQ; using carefully purified recombinant proteins and quantitative methods to study protein interactions, we recently demonstrated that the presence of an expanded polyQ does not per se alter the interactions of a monomeric mutant protein with its partners (12); finally, anti-polyQ antibodies such as MW1 and 1C2 were shown to recognize short polyQ stretches, and not a mutant-specific conformation (10,14,21).

Although the detailed characterization of the 3B5H10 antibody has only been published quite recently (20,22), it has
A number of findings indicate that non-pathogenic and pathogenic polyQ share many biophysical and structural properties: they are intrinsically disordered (10,11,14,15,33) but rather compact (35,36) as soluble monomers, and they undergo similar conformational changes, leading to the formation of aggregates with overall similar amyloid-like properties and structural features (11,17,37–39). However, some properties of polyQ vary with polyQ length: for instance, progressive polyQ-length-dependent variations were reported in the mature aggregates, such as differences in the size and shape of the fibres, in their exhibitin of Congo Red birefringence (37) or in their ability to form intramolecular contacts (39); along the same line, the nucleation process and the aggregation kinetics are influenced by polyQ length variations (11,40). It is now crucial to decipher whether and how such polyQ-length-dependent variations contribute to trigger nine neurodegenerative diseases, intrinsically caused by polyQ elongation.

MATERIALS AND METHODS

Protein expression and purification

The 1C2 antibody (IgG1) from mice ascitic fluids was purified using a single affinity purification step against a GST-Q11 fusion protein pre-bound on a Glutathione-sepharose column. Treatment with thrombin protease, on the column, in phosphate 50 mM pH 7 buffer, allowed for release of the Q11:1C2 complex off the column. The Q11 peptide was removed by dialysis (cut-off 50 kDa), and the pure 1C2 was then cleaved by papain treatment as reported earlier (14). Fc fragments were retained by protein-A sepharose beads and 1C2-Fab retrieved pure in the supernatant as reported (14). 1C2-Fab was either dialyzed in ammonium acetate (50 mM) for ESI-TOF analysis, or in various buffers for crystallization (10 mM Tris, 10 mM NaCl, pH 7.3) or SPR [10 mM HEPES, pH 7.4, 150 mM NaCl, 3.4 mM EDTA, 0.005% (v/v) Tween20; (P685 Sigma-Aldrich)]. 1C2-Fab was stored at 4°C at concentration ~10–20 μM and concentrated on Amicon Ultra systems (Millipore) at up to 200 μM (~10 mg/ml) for crystallization purposes. 1C2 and 1C2-Fab production are summarized in Supplementary Material, Figure S1a. 3B5H10 was purchased from SIGMA-ALDRICH (1874). 3B5H10-Fab was prepared following methods identical to that reported for 1C2-Fab (14). GST-polyQ proteins were purified as reported (14).

Crystallization and structural resolution methods are detailed in Supplementary Material, Materials and Methods and Figures S1b–d and S2.

GST pull-down assays

Phosphate buffer 50 mM pH 7 (4°C) was used at all steps. Three microlitres of prewashed and equilibrated Glutathione-sepharose beads (GE 17-0756-01) were loaded with equal quantities of GST (negative control), GST-Q11, GST-Q22 or GST-Q41 proteins (270 μM at 80 μM), washed (10 column volumes) and kept at 4°C as sources of GST and GST-polyQ beads for 4 days (time needed to perform three GST pull-down experiments). To realize the pull-down while using as little 1C2 or 3B5H10 IgGs as possible, only 10 μl of either GST or GST-polyQ beads was loaded on home-made micro-columns

Figure 3. Structural insights into MW1, 1C2 and 3B5H10 interactions with small polyQ in a linear and extended conformation. (A) Representation of the MW1:Q10 interaction surface [pdb code 2OTU, (21)]. The Q10 peptide (yellow/orange) adopts an extended conformation and sits diagonally in a shallow groove on the surface of the Lvαx (green) and Hv (blue) MW1 subunits. The amino acids of the Lv moiety in contact with the polyQ peptide (21) are coloured in dark green. The glutamine side chains of the polyQ are displayed as thin lines, except two of them that are shown as sticks: the yellow one (N’4) that mediates canonical contacts with the Lvαx domain [26] and Fig. 1F and the orange one (N’6) that mediates six contacts with the Lvαx domain, and only one with the Hv domain, representing the limit of the polyQ peptide in contact with the Lvαx domain. The approximate positions of the loops L1, L3, L1 and H3—that contact the polyQ peptide—are indicated. The same codes (colour and representation) apply in (B)–(D). (B) Representation of the Fv domain of apo MW1 [pdb code 2GSG, (21)], on which the polyQ peptide was positioned as observed in the MW1-Q10 structure (A): the structure of the apo Fv domain of MW1 was aligned on the apo MW1 structure. In this position, the polyQ peptide is closer to H3 (steric clash) and further away from L3, when compared with the MW1:Q10 structure (A). Indeed, it was reported that upon polyQ binding, major structural changes involve H3, which moves to avoid direct contacts with the glutamine No. 9, and L3 which moves towards the polyQ peptide (21). These structural changes observed upon polyQ binding are indicated by two red arrows. (C and D) Representation of the Fv domains of 1C2 (C) and 3B5H10 (D), on which a polyQ peptide Q6 was positioned on the Lvαx surface, as observed in the MW1-Q10 structure (A): the structures of the Fv domains of 1C2 (C; PDB code 4ISV) and 3B5H10 (D; PDB code 3S96) were aligned with that of MW1 in complex with Q6, and the Q10 peptide was superimposed on the apo MW1 structure. In this position, the polyQ peptide is closer to H3 (steric clash) and further away from L3, when compared with the MW1:Q10 structure (A). Structural changes similar to those occurring for MW1 [as shown in (B)] are also likely to occur upon polyQ binding on 1C2 and 3B5H10.
Surface plasmon resonance

The instrumentation and methodologies were adapted from Klein et al. (14) and Davranche et al. (12). The interaction studies were carried out in real time by SPR analysis using a Biacore T200 instrument (GE Healthcare Biacore, Uppsala, Sweden). Experiments were carried out at 25 °C using a flow rate of 30 μl/min and HBS as running and dilution buffer [10 mM HEPES, pH 7.4, 150 mM NaCl, 3.4 mM EDTA, 0.005% (v/v) Tween20; (P6585 Sigma-Aldrich)].

To capture GST-Qn molecules in Figure 2B, anti-GST antibody (GST capture kit BR100838 GE Healthcare Biacore) was directly immobilized to all four flow cells of the chip according to the supplier’s protocol (GE Healthcare). One flow cell was used as a control and was loaded with GST (reference surface) and the other three flow cells were loaded with GST-Q11, GST-Q22 or GST-Q41 (14).

For the experiment described in Figure 2D, carboxymethylated dextran sensor chips sCM5 (BR-100030 GE Healthcare Biacore) were used to immobilize purified GST (reference surface), GST-Q11 and MBP-Htt-exon1-Q16 proteins, via the standard amine coupling method (amine coupling kit, BR-100050 GE Healthcare Biacore).

Serial dilutions of each purified Fab or Mab were injected over the four cells at a flow rate of 30 μl/min. Each cycle consisted of an 80 or 120 s sample injection followed by a post-injection phase of 7200 s in running buffer.

Correction of all binding curves was performed by so-called double referencing, i.e. subtraction of the data of the GST control flow cell followed by subtraction of the data from an average of three buffer injections (Myszka, 2000).

The initial reaction rate on the GST-polyQ surfaces was not affected by flow variations between 5 and 100 μl/min (GST-polyQ immobilization levels less than 120 RU and 20 nm injected 1C2 and 3B5H10-Fab’s or Mab’s), indicating the absence of mass-transport phenomenon.

To determine Fabs- and Mabs-binding affinities and/or kinetic parameters for the different Qn antigens, the data from six different concentrations were globally fitted to molecular interaction models found in Biacore T200 evaluation software [1 to 1 binding, bivalent analyte (A + B ⇔ AB; AB + B ⇔ AB2); heterogeneous ligand (A + B1 ⇔ AB1; A + B2 ⇔ AB2); and two-state reaction (A + B ⇔ AB ⇔ AB*)]. Regression analysis of kinetic data was limited to these relatively simple molecular interaction models which provided acceptable fits in order to compare the affinity orders even when experimental data are complex. All tested models provided consistent results, i.e. micromolar affinities. The results for the ‘1 to 1 binding mode’ are shown in Supplementary Material, Fig. S4b–c.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG online.

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(pipette tips plugged with cotton). Equal quantities of either 1C2 or 3B5H10 (15 μl at 5 μM) were loaded on GST, GST-Q11, GST-Q22 or GST-Q41 columns. After the IgGs had passed through the column and after washing (8 column volumes), the beads were resuspended in 15 μl of SDS/PAGE running buffer containing Laemmli loading dye, boiled for 2 min and loaded on SDS/PAGE (16%) for electrophoresis.

WB interaction assays of 1C2 and 3B5H10 with GST-polyQ proteins

Equal amounts of pure GST-Q11, GST-Q22 and GST-Q41 proteins (200 picomole each) were mixed in 150 μl SDS/PAGE running buffer containing Laemmli loading dye, and boiled for 2 min. They were loaded on a unique and 6.2 cm wide SDS/PAGE (16%) gel. After electrophoresis and transfer on nitrocellulose membrane, the unique lane was cut into 15 equivalent lanes. Each ~4 mm wide lane was individually incubated for 90 min at room temperature (RT) in 2 ml of different primary antibodies: purified 1C2 or 3B5H10 IgGs, or purified 1C2-Fab or 3B5H10-Fab, or home-made monoclonal anti-GST ascites (dilution 1/60 000). The concentrations of primary antibodies and identity of the lanes shown are Supplementary Material, Fig. S4b–c.
REFERENCES


