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Properties of polyglutamine expansion *in vitro* and in a cellular model for Huntington's disease

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Eight neurodegenerative diseases have been shown to be caused by the expansion of a polyglutamine stretch in specific target proteins that lead to a gain in toxic property. Most of these diseases have some features in common. A pathological threshold of 35–40 glutamine residues is observed in five of the diseases. The mutated proteins (or a polyglutamine-containing subfragment) form ubiquitinated aggregates in neurons of patients or mouse models, in most cases within the nucleus. We summarize the properties of a monoclonal antibody that recognizes specifically, in a Western blot, polyglutamine stretches longer than 35 glutamine residues with an affinity that increases with polyglutamine length. This indicates that the pathological threshold observed in five diseases corresponds to a conformational change creating a pathological epitope, most probably involved in the aggregation property of the carrier protein. We also show that a fragment of a normal protein carrying 38 glutamine residues is able to aggregate into regular fibrils *in vitro*. Finally, we present a cellular model in which the induced expression of a mutated full-length huntingtin protein leads to the formation of nuclear inclusions that share many characteristics with those observed in patients: those inclusions are ubiquitinated and contain only an N-terminal fragment of huntingtin. This model should thus be useful in studying a processing step that is likely to be important in the pathogenicity of mutated huntingtin.

Keywords: polyglutamine expansion; aggregation; cellular model; nuclear inclusion; pathological epitope; Huntington's disease

1. INTRODUCTION

Huntington's disease (HD) and seven other neurodegenerative diseases are caused by CAG codon/polyglutamine (polyQ) expansions that lead to a gain in toxic properties in target proteins (reviewed by Paulson & Fischbeck (1996) and Wells & Warren (1998)). In five of these diseases the pathological threshold is between 35 and 40 glutamine residues, whereas it is higher for dentatorubral–pallidoluysian atrophy (DRPLA) (49 glutamine residues) and for spinocerebellar ataxia 3 (SCA3) (61 glutamine residues) (Wells & Warren 1998). In SCA6, the pathological polyQ is much smaller (21 glutamine residues); the overlap of clinical manifestations with those caused by the conventional loss of function mutations within the same gene suggests that in this case the polyQ expansion could instead cause an alteration of the normal function (Zoghbi 1997). Recent studies reviewed in this issue have shown that proteins containing pathological polyQ stretch can form ubiquitinated aggregates within neurons of patients and of some transgenic mouse models (Davies *et al*. 1997; Skinner *et al*. 1997; Paulson *et al*. 1997; DiFiglia *et al*. 1997; Becher *et al*. 1998; Igarashi *et al*. 1998). In many cases the aggregates were found in the nucleus, despite the fact that the cognate normal protein is cytoplasmic (as for huntingtin or ataxin-3). For huntingtin, the protein that is found in nuclear inclusions or in dystrophic neurites corresponds to an N-terminal fragment of huntingtin that contains the polyQ expansion (DiFiglia *et al*. 1997). There is also evidence that in SCA3 only a subfragment containing the polyQ stretch is present in nuclear inclusions (Paulson *et al*. 1997). Studies of transgenic mouse and in transfected cellular models have also shown that truncated proteins containing a pathological polyQ stretch are much more toxic, or have increased aggregation properties, than the full-length mutated proteins (Davies *et al*. 1997; Paulson *et al*. 1997; Igarashi *et al*. 1998; Martindale *et al*. 1998; Merry *et al*. 1998; Cooper *et al*. 1998; Li & Li 1998; Lunkes & Mandel 1998; Klement *et al*. 1998; Saudou *et al*. 1998; Butler *et al*. 1998). This suggests that proteolysis of polyQ-containing proteins and their aggregation might be important steps in pathogenesis.

Here we present work from our laboratory on two major aspects. We summarize the properties of the 1C2

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antibody that recognizes specifically, in a Western blot, proteins carrying a pathological polyQ expansion (Trottier *et al*. 1995). This indicates that in the range of 35–40 glutamine residues, which corresponds to the pathological threshold in five of the polyQ diseases, a change in conformation occurs that strengthens the interaction with 1C2 antibody. This conformational epitope is most probably involved in the aggregation properties demonstrated *in vitro* or *in vivo*, notably by mutated huntingtin (Davies *et al*. 1997; Scherzinger *et al*. 1997). We observed that even the normal stretch of 38 glutamine residues carried by the human transcription factor TBP, which is recognized by the 1C2 antibody, is able to aggregate into regular fibrils similar to those observed by Scherzinger *et al*. (1997) for the N-terminal subfragment of mutated huntingtin.

To decipher some of the cellular events that link the processing of a target protein with expanded polyQ and its aggregation to neuronal dysfunction and ultimately to death, cellular models are highly desirable. Here we present a cellular model in which the expression of full-length huntingtin with polyQ expansion can lead to the formation of nuclear inclusions that seem similar to those found in patients, notably in that they contain only a short N-terminal fragment (Lunkes & Mandel 1998). This model should therefore be useful in analysing the processing step necessary for building up nuclear inclusions.

2. CONFORMATIONAL EPITOPE AND AGGREGATION OF POLYQ TRACTS

(a) *Recognition properties of 1C2 monoclonal antibody reveal a pathological epitope*

The 1C2 monoclonal antibody was initially developed by using as antigen the human TATA-binding protein (TBP), a general transcription factor that contains a polymorphic polyQ stretch with the most common allelic form of 38 glutamine residues, and rare variants having 42 glutamine residues (Imbert *et al*. 1994; Gostout *et al*. 1993). Epitope mapping of the antibody indicated that it recognizes the polyQ domain of TBP (Lescure *et al*. 1995). We found that the 1C2 antibody shows the unexpected property of selectively recognizing pathological polyQ expansions in the proteins involved in HD, SCA1 and SCA3 (Trottier *et al*. 1995). A Western blot analysis of allelic forms of huntingtin showed that the intensity of detection by the 1C2 antibody is dependent on the size of the expansion, increasing from 39 to 85 glutamine residues. In contrast, huntingtin with a normal polyQ stretch was not detected, whereas allelic forms with polyQ at the upper normal range (28–33 glutamines) were faintly detected after a very long exposure of the blot. Thus, the efficiency of detection seemed to parallel the severity of the disease.

The recognition properties of the 1C2 antibody could be explained by a higher affinity for a pathological expansion, owing to the presence of a specific conformational epitope that appears when the polyQ is more than 35 residues long. Alternatively, or synergistically, the repetition of a polyQ epitope could facilitate a bivalent binding of the antibody and thus stabilize the interaction.

To study the binding properties between 1C2 antibody and polyQ stretches, we have performed kinetic analyses of the interaction under native conditions with a surface plasmon resonance biosensor (BIAcore™, Pharmacia) (Myszka 1997). The Fab (which has a single antigen-binding site) showed a much stronger affinity for an expansion of 73 glutamine residues than for a normal polyQ stretch (15 glutamine residues). The complex formed between the Fab and the expansion to 73 glutamine residues is remarkably stable, and dissociates at 1/100 of the rate found with the stretch of 15 glutamine residues (Trottier *et al*. 1998). Similar kinetics of interaction were obtained with the whole antibody, indicating that a bivalent binding is not a major factor in the detection process. The results indicate the formation of a novel conformation by an expanded polyQ stretch.

The 1C2 antibody was used to test the hypothesis of polyQ expansion as the cause of several neurological diseases that show an anticipation phenomenon. On Western blot analysis, the 1C2 antibody was able to detect novel proteins containing polyQ expansion in patients with autosomal dominant spinocerebellar ataxia type 2 (linked to chromosome 12) and type 7 (linked to chromosome 3) (Trottier *et al*. 1995). Another protein has been recently detected by the 1C2 antibody in a single patient with autosomal dominant familial spastic paraplegia (AD-FSP) linked to chromosome 2, which also showed a specific repeat expansion detection (RED) product (Nielsen *et al*. 1998). However, in the AD-FSP patients that we studied (some were linked to chromosome 2), we were unable to detect novel protein with the 1C2 antibody (Trottier *et al*. 1995). Thus, the implication of polyQ expansion in this disease needs to be confirmed. Several groups have tried without success to identify new types of polyQ expansion in patients with sporadic or unassigned autosomal dominant cerebellar ataxia (Stevanin *et al*. 1996; Lopes-Cendes *et al*. 1996). One should, however, note that although a positive result proves the mechanism, false negative findings can be obtained if the pathological protein is poorly expressed in, or absent from, the cells analysed, or if the polyQ stretch is too small to be detected with high sensitivity. In the search for a putative polyQ expansion protein, the analysis of early-onset cases is warranted, given the strong polyQ length-dependent properties of the antibody (Trottier *et al*. 1998).

Anticipation was also reported to occur in some families with bipolar affective illness or schizophrenia, suggesting a possible implication of polyQ expansion (Ross *et al*. 1993). The detection of CAG repeats by using the RED method has not given a clear indication for such an involvement. Several groups have undertaken studies with the use of 1C2 antibody to search for pathological polyQ expansion in lymphoblasts from schizophrenia patients.

The 1C2 antibody was used to screen two cDNA expression libraries from SCA2 and SCA7 lymphoblasts, respectively. No clones corresponding to the expected mutated alleles were recovered in these libraries, possibly owing to a toxic effect of expanded polyQ in *Escherichia coli*, as reported by Onodera *et al*. (1996). We did, however, observe that 1C2 was able to detect smaller polyQ repeats (more than 11 glutamine residues) in the bacteriophage plaque screening conditions, which are less

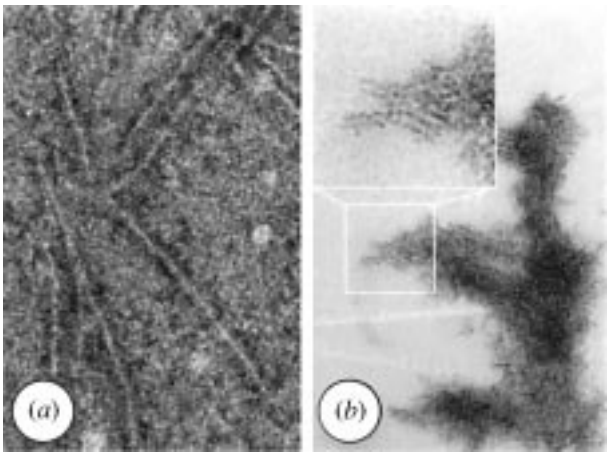

Figure 1. Aggregation of a N-terminal fragment of TBP with 38 glutamine residues. (*a*) The N-terminal domain of the human TBP (154 residues) with 38 glutamine residues in fusion with a polyhistidine tag was expressed in *E. coli* and purified on a nickel chelating column and by gel filtration. Aliquots of freshly purified N-TBP fragments, kept in 100 mM Hepes, pH 7.2, at a concentration of 1 mg ml^{-1}, were diluted to a final concentration of 30 µg ml^{-1}, deposited on a glow-discharged electron microscopy grid covered with a thin carbon film and negatively stained with a 20 g l^{-1} uranyl acetate solution. Filaments *ca.* 0.1–0.5 µm in length and 10–12 nm wide were clearly observed. Filaments similar in size were also observed when the polyhistidine tag was removed enzymically, thus demonstrating that the tag does not influence the aggregation process (results not shown). The bar represents 100 nm. (*b*) On longer storage (three or four days), the N-TBP fraction formed large aggregates composed of filaments similar to those described in (*a*). The scale bar represents 200 nm and the insert is enlarged twice to show the filamentous organization.

stringent than for Western blotting (no SDS or reducing agent). This allowed us to identify five new polyQ coding cDNA species, including one corresponding to a normal allele of the *SCA2* gene (Imbert *et al.* 1996) and another corresponding to a Ca^{2+}-regulated K^+ channel gene (*hSKCa3*) (Wittekindt *et al.* 1998).

The striking parallel between the effect of increased polyQ length in disease severity and in recognition by 1C2 antibody suggests that the novel conformational epitope recognized by this antibody has a key role in pathogenicity, possibly by favouring aggregation and/or by altering interaction with other cellular proteins. Determination of the crystal structure of expanded polyQ is an important goal, but is rendered difficult by the great tendency of polyQ-carrying polypeptides to aggregate *in vitro*.

(b) *Aggregation of a normal fragment of TBP containing 38 glutamine residues*

The alleles of TBP carrying 38–42 glutamine residues are larger than the pathological threshold of 35–37 glutamine residues observed in HD, SCA2 and SCA7. These allelic TBP proteins are well recognized by 1C2 antibody; however, they are not pathogenic proteins. We intended to use an N-terminal fragment of TBP with a stretch of 38 glutamine residues (N-TBP-38Q) for X-ray crystallography. After overexpression in *E. coli*, the purified N-TBP-38Q showed a tendency to aggregate as seen on non-denaturing gel electrophoresis (results not shown). When freshly purified N-TBP-38Q fractions were investigated by electron microscopy, we observed long filaments (0.1–0.5 µm) that were 10–12 nm in diameter (figure 1*a*), very similar to those reported by Scherzinger *et al.* (1997) with a subfragment of huntingtin carrying 51 glutamine residues. The filaments that we observed were not uniformly distributed over the entire surface but were organized in clusters of three to a few tens of filaments, suggesting that they interact or that their mechanism of growth promoted the formation of discrete branching points, thus resulting in a loose network. The number of such structures increased with time during the two days after purification, whereas their number decreased after three days, a time that corresponded to the appearance of large three-dimensional aggregates composed of a dense irregular filamenteous network (figure 1*b*). The aggregation of N-TBP-38Q *in vitro* precludes the formation of crystal. Interestingly, the preincubation of N-TBP-38Q with the Fab fragment of 1C2 antibody prevents filament formation and aggregation. Instead we observed small complexes formed of two or three stain-excluding domains the size of which was consistent with that of a Fab fragment (results not shown). The observation that interaction with 1C2 antibody prevents such an aggregation led us to initiate attempts to co-crystallize N-TBP-38 glutamine residues or a pathological ataxin-2 fragment (42 glutamine residues) with the Fab fragment of 1C2 antibody.

The stretch of 38 glutamine residues present in TBP can lead to the formation of fibrils of very similar appearance to those found with the N-terminal fragment of huntingtin (Scherzinger *et al.* 1997). In our case, the fact that an anti-polyQ antibody blocked the aggregation indicated the importance of the polyQ stretch for the formation of supramolecular assemblies. It is likely that the folding of full-length TBP prevents this aggregation from occurring *in vivo* (especially because the nuclear localization of this protein would probably favour toxicity). Thus, the variability in pathological threshold could be dependent on both the folding of each full-length target protein and on the proteolytic activities that lead to the production of more toxic subfragments.

3. A CELLULAR MODEL FOR HUNTINGTON'S DISEASE

(a) *Formation of inclusions in cells expressing mutant huntingtin*

In none of the previous cellular models designed for Huntington's disease could the situation *in vivo* be simulated by the expression of full-length mutant protein. We have now established a regulated system for the expression of huntingtin under the control of the reverse tetracycline-inducible transactivator (Gossen *et al.* 1995), in the mouse–rat neuroblastoma–glioma cell line NG108-15. In this system, the addition of doxicycline to the culture medium induces the expression of huntingtin, thus preventing potential toxic effects of overproduced mutant huntingtin when growing the cells. The NG108-15 cells display neuronal-like properties after differentiation (Nelson *et al.* 1976), and they allow the study of the expression of mutant huntingtin over much longer durations (up to 18 days) than in transient transfection systems described previously (Martindale *et al.* 1998; Cooper *et al.* 1998; Li & Li 1998).

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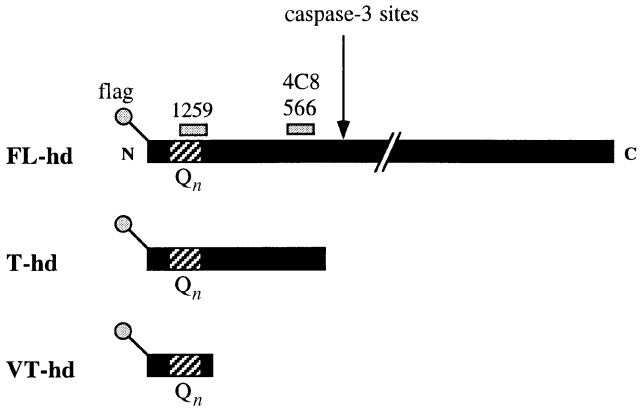

Figure 2. Features of the huntingtin-derived proteins. The full-length and truncated huntingtin cDNA constructs with different lengths of CAG repeats were flag-tagged at the N-terminus and cloned into the pUHD172-1neo vector (Gossen & Bujard 1992; Gossen *et al*. 1995). The hatched boxes correspond to a polyQ repeat with $n = 15$, 73 or 116. The localization of the epitopes (grey boxes) of the antibodies used, and the predicted caspase-3 cleavage sites, are indicated.

In a first step, the reverse tetracycline-inducible transactivator (rtTA) was stably integrated in the cell line NG108-15. To study the fate of full-length huntingtin compared with truncated forms, as well as the effect of polyQ length, double-transformed cell lines expressing the following proteins under the control of the tetracycline transactivator promoter were then generated: full-length (FL) huntingtin with 15, 73 and 116 repeats; truncated (T) huntingtin (502 residues) with 15 and 73 repeats, and a very truncated (VT) huntingtin protein (80 residues) with 15, 73 and 120 repeats (figure 2). All these proteins carry the flag-tag epitope at their N-terminus. The T-form is similar to the N-terminal fragment generated by cleavage at the caspase-3 site (Goldberg *et al*. 1996), whereas the VT form is similar to that expressed in transgenic mice studied by Mangiarini *et al*. (1996) and Davies *et al*. (1997).

Huntingtin expression was assessed by immunofluorescence after differentiation and induction with doxicycline, by using the anti-flag monoclonal antibody (mAb). Homogeneous cytoplasmic staining was observed in all lines expressing the normal huntingtin constructs. In contrast, distinct intracellular inclusions (cytoplasmic inclusion, (CI) or nuclear inclusion (NI)) were seen in the mutant lines T-hd73, FL-hd73 and FL-hd116, coexisting in most instances with homogeneous cytoplasmic staining (Lunkes & Mandel 1998). Both types of inclusion (NIs and CIs) also reacted with an antibody against ubiquitin, whereas the NIs were also detected with an antibody against the nuclear protein SUG1, which is a subunit of the PA700 complex of the proteasome (Fraser *et al*. 1997) (figure 3). Those observations most probably reflect failed attempts of the cell to remove misfolded or aggregated proteins. In line VT-hd73, expressing the shortest huntingtin protein, no homogeneous cytoplasmic staining was observed. Instead the protein seemed to aggregate as dense inclusions, either in the cytoplasm (CI) or in the nucleus (NI). In a time-course experiment, the frequency of CIs decreased from 49% (day 4) to 4% (day 16), whereas the frequency of NIs increased from 20% (day 4) to 84% (day 16) (figure 4), suggesting that the CIs might be precursors of the NIs. Cells expressing the T-hd73 truncated construct showed a delayed and less efficient formation of dense NIs (up to 17%), and for FL-hd73 only 1% of expressing cells formed NIs at late time points (16 days) (Lunkes & Mandel 1998). A much greater formation of small dense NIs was observed in line FL-hd116, starting at around 12 days of induction, whereas CIs predominated at earlier time points (figure 5). The NIs were detected with the anti-flag mAb but not with the 4C8 mAb or the 566 polyclonal antibody (the epitopes of which are just proximal to the caspase-3 sites) (Lunkes & Mandel 1998). This suggests that these NIs are composed of a fragment cleaved N-terminal to the 4C8 epitope, which is in agreement with the finding that inclusions in the brain of HD patients are detected only with N-terminal antibodies close to the polyQ stretch (DiFiglia *et al*. 1997).

The dense nuclear inclusions were not stained by the 1C2 mAb that is specific for expanded polyQ tracts (Trottier *et al*. 1995). Most probably, the pathological epitope is masked in the dense aggregates, possibly owing to the interaction of polyQ tracts with each other to form β-pleated sheets (Perutz *et al*. 1994). This is consistent with the lack of staining of inclusions by 1C2 in transgenic HD mice (Davies *et al*. 1997), with its very weak reaction with nuclear inclusions seen in the brain of SCA3 patients (Paulson *et al*. 1997), and with its failure to detect aggregates of the mutant androgen receptor on a Western blot (Merry *et al*. 1998).

(b) *Processing of mutant full-length huntingtin*

To study the processing of the huntingtin protein, we performed a Western blot analysis of cells expressing mutant full-length huntingtin. A breakdown product of *ca*. 98 kDa for FL-hd116 was detected from day 10 onwards by both the anti-flag mAb (not shown) and the 4C8 mAb (figure 6). The size of the fragment corresponded well to that of the predicted N-terminal caspase-3 cleavage product of huntingin (Goldberg *et al*. 1996), which includes the 4C8 epitope. Although endogenous huntingtin is produced at levels similar to that of mutant huntingtin (as detected by the 4C8 mAb), the corresponding caspase-3 fragment was not observed in these lines, which is consistent with the hypothesis that an elongated polyQ repeat enhances the cleavage of full-length huntingtin by caspase-3 (Goldberg *et al*. 1996). However, although inclusions were detectable at days 4–6 (figure 5), the potential caspase-3 fragment appeared rather later (day 10). This suggests that this fragment is not involved in the initial formation of inclusions. However, we could not observe in our Western blot analysis the shorter fragment predicted from the immunofluorescence studies of inclusions, most probably owing to the high aggregation potential of such a fragment.

(c) *Conclusion*

Cells expressing full-length mutant huntingtin show the formation of cytoplasmic and nuclear inclusions in a manner dependent on time and polyQ length. These inclusions are ubiquitinated and contain only a short N-terminal part of mutant huntingtin, thus appearing similar to those observed in the brain of HD patients

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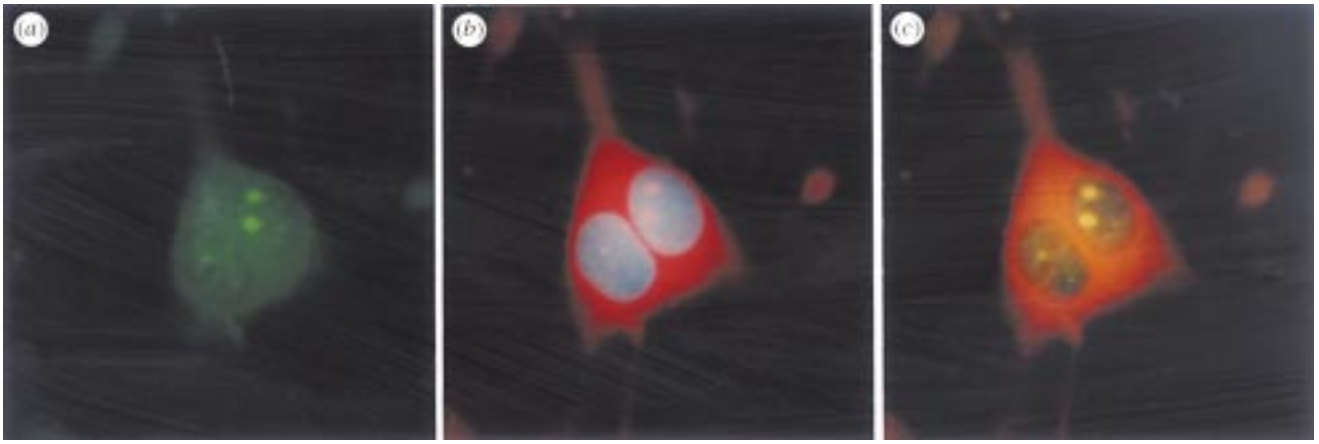

Figure 3. Co-localization of N-terminal huntingtin and SUG1, a subunit of the PA700 complex of the proteasome, in inclusions observed in NG108-15 cells expressing FL-hd116. Cytoplasmic huntingtin and NIs are detected with the N-terminal polyclonal antibody 1259 (produced against a glutathione S-transferase fusion protein containing residues 1–171 of huntingtin (D. Devys, unpublished data)) (*b*). The anti-SUG1 mAb (Baur *et al*. 1996) reacts strongly with inclusions in the nucleus (*a*), which co-localize with the huntingtin-positive NIs (*c*). The co-localization has been confirmed with antibodies raised against known (such as Mss1 (Fraser *et al*. 1997)) and unknown proteins found enriched in a PA700-containing complex of high molecular mass (Y. Lutz, personal communication) (A. Lunkes, unpublished data).

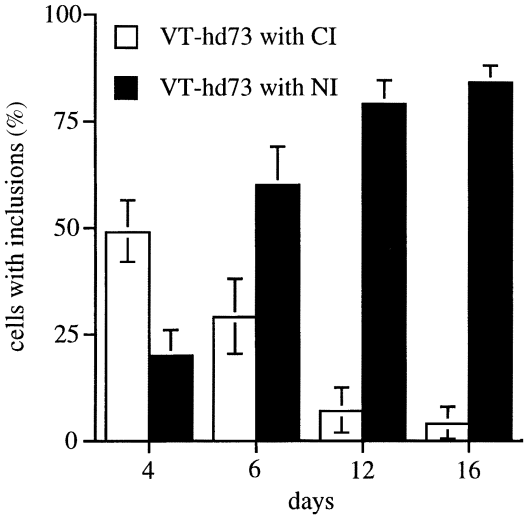

Figure 4. Fate of the VT-hd73 transgene protein. The frequency of cells bearing cytoplasmic inclusions (CI) decreased over time, whereas the inclusions in the nucleus (NI) increased. The results are from one experiment. Similar results were obtained with cells expressing the VT transgene with 120 glutamine residues. Bars represent the s.e.m. of the proportion of cells having cytoplasmic or nuclear inclusions.

(DiFiglia *et al*. 1997). This indicates that a processing step generating fragments with high aggregation potential operates in these cells. We are currently mapping the exact site of cleavage as a step towards the identification of the protease involved, which could constitute an important factor in the pathogenesis of HD, and thus a potential therapeutic target.

The cellular models presented so far for polyQ expansion diseases were based on transient transfections (Paulson *et al*. 1997; Igarashi *et al*. 1998; Martindale *et al*. 1998; Butler *et al*. 1998; Cooper *et al*. 1998; Li & Li 1998). In those short-duration assays the formation of cytoplasmic inclusions was observed only in cells expressing truncated mutant forms, or at low level in cells expressing a full-length huntingtin with 128 glutamine residues,

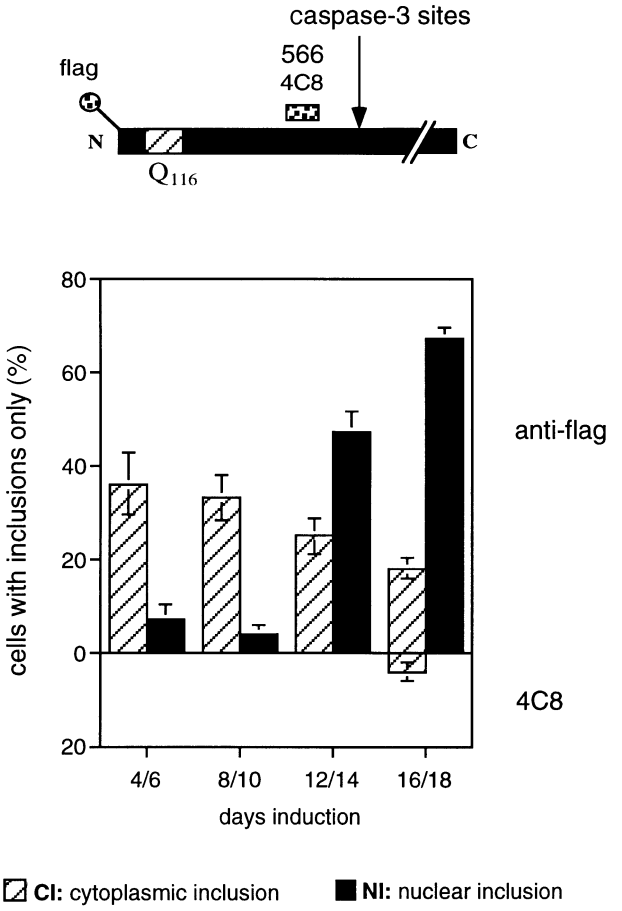

Figure 5. Formation of inclusions in a cell line expressing full-length huntingtin (FL-hd116). Cells expressing full-length huntingtin with tracts of 116 glutamine residues were differentiated and induced with doxicycline. Immunofluorescence analysis was performed with the anti-Flag and 4C8 mAbs. The 4C8 mAb detected only a low frequency of CIs at late stages, and no NIs. The results of one representative experiment are shown.

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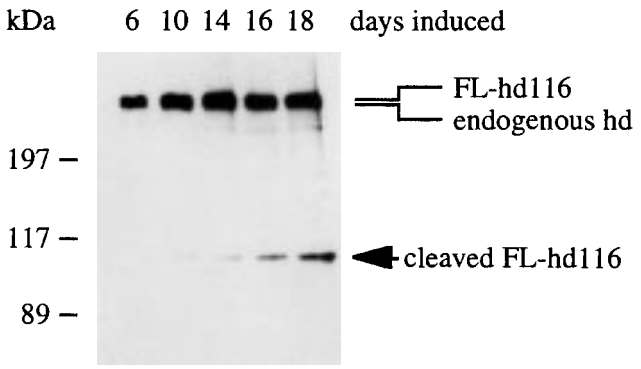

Figure 6. Cleavage of the mutant full-length huntingtin protein. Cells expressing FL-hd116 were differentiated and induced with doxicycline over 18 days; samples were taken at indicated time points for Western blotting. Immunoprobing with the huntingtin-specific mAb 4C8 revealed, from day 10 onwards, a breakdown product corresponding to the size of the putative caspase-3 fragment. No cleavage product was detected for the endogenous huntingtin (also detected by the 4C8 mAb), which was expressed at about the same level as the mutant transgene protein.

whereas NIs were detected only in cells expressing a very short form of mutant huntingtin (exon-1 with 128 CAG repeats) (Martindale *et al*. 1998).

The model presented here provides a tool for the elucidation of the mechanisms leading to the cleavage of full-length huntingtin, its role in the formation of intracellular aggregates, and the effects on the latter on cellular physiology. It will be especially interesting to see whether in this system protease inhibition or transglutaminase inhibition might interfere with the formation of inclusions and their consequences on cell function and survival.

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