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THE ROYAL SOCIETY

Analysis of the subcellular localization of huntingtin with a set of rabbit polyclonal antibodies in cultured mammalian cells of neuronal origin: comparison with the distribution of huntingtin in Huntington's disease autopsy brain

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Huntington's disease (HD) is a neurodegenerative disorder with a midlife onset. The disease is caused by expansion of a CAG (glutamine) repeat within the coding region of the HD gene. The molecular mechanism by which the mutated protein causes this disease is still unclear. To study the protein we have generated a set of rabbit polyclonal antibodies raised against different segments of the N-terminal, central and C-terminal parts of the protein. The polyclonal antibodies were affinity purified and characterized in ELISA and Western blotting experiments. All antibodies can react with mouse and human proteins. The specificity of these antibodies is underscored by their recognition of huntingtin with different repeat sizes in extracts prepared from patient-derived lymphoblasts. The antibodies were used in immunofluorescence experiments to study the subcellular localization of huntingtin in mouse neuroblastoma N1E-115 cells. The results indicate that most huntingtin is present in the cytoplasm, whereas a minor fraction is present in the nucleus. On differentiation of the N1E-115 cells *in vitro*, the subcellular distribution of huntingtin does not change significantly. These results suggest that full-length huntingtin with a normal repeat length can be detected in the nucleus of cycling and non-cycling cultured mammalian cells of neuronal origin. However, in HD autopsy brain the huntingtin-containing neuronal intranuclear inclusions can be detected only with antibodies raised against the N-terminus of huntingtin. Thus several forms of huntingtin display the propensity for nuclear localization, possibly with different functional consequences.

Keywords: Huntington's disease; antibody characterization; huntingtin; subcellular localization; neuronal differentiation

1. INTRODUCTION

Huntington's disease (HD) is a neurodegenerative disorder with a midlife onset. The neuropathology involves a loss of neurons in specific parts of the brain, with the striatum being most severely affected. The biochemical basis of the disease is not understood and so far no cure is available.

The gene involved in Huntington's disease encodes a 348 kDa protein called huntingtin. The mutations found in patients imply that HD belongs to the so-called triplet repeat expansion diseases; a $(CAG)_n$ repeat coding for a polyglutamine stretch is expanded in HD patients. The normal range of the repeat sizes is between 11 and 36 units, whereas patients have 37–121 copies (Huntington's Disease Collaborative Research Group 1993; De Rooij *et al*. 1993; Read 1993). In patients, both alleles (normal and mutated) are expressed (Jou & Myers 1995), suggesting a gain of function of the mutant protein. In this model, the expanded polyglutamine part of the protein confers a new property that disturbs the normal activity of huntingtin or causes an interaction with novel partners. In line with the latter proposal, a protein, designated Hapl, has been identified that interacts preferentially with huntingtin with an extended repeat (Li *et al*. 1995). However, another protein has been identified, Hipl, that binds preferentially to normal-sized huntingtin (Kalchman *et al*. 1997; Wanker *et al*. 1997).

The suggestion that the observed alteration of the CAG-encoded polyglutamine stretch of huntingtin in HD

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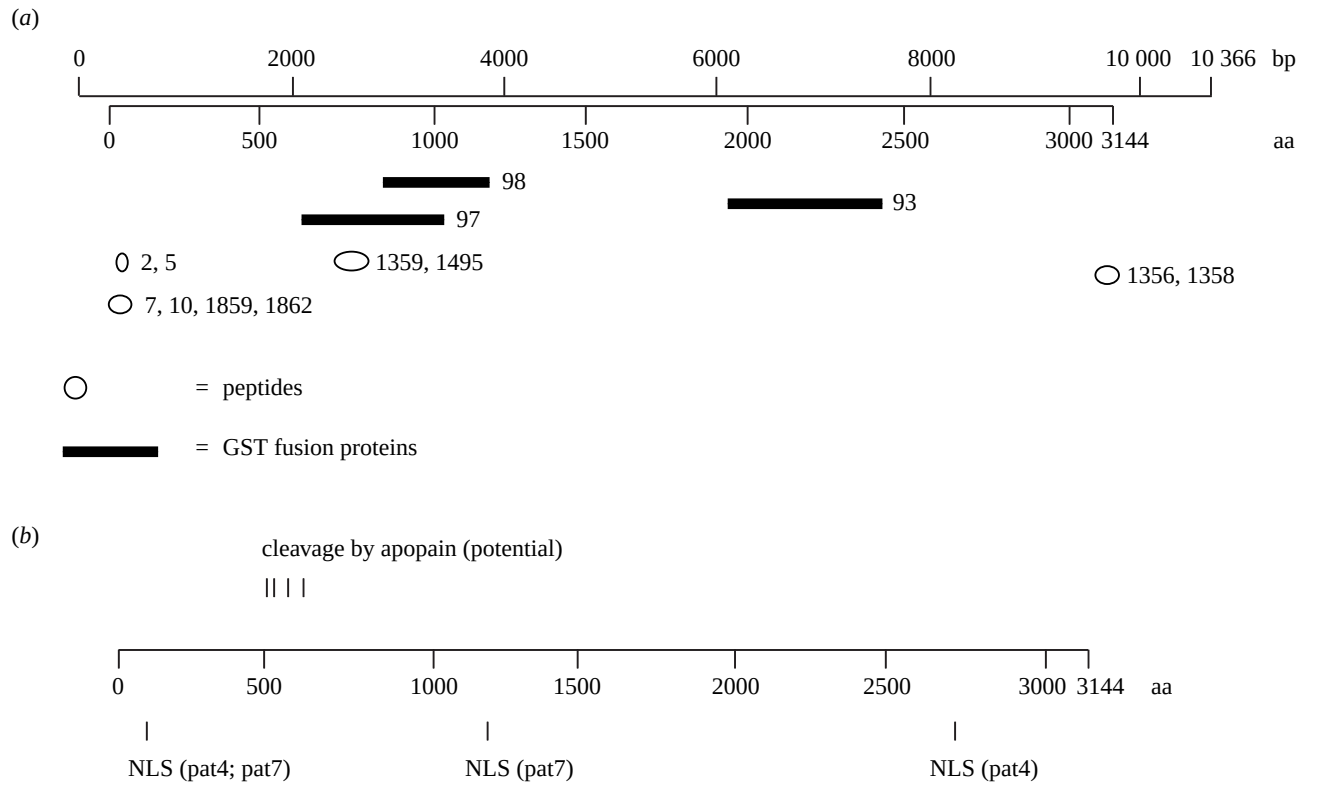

Figure 1. The localization of the antigens used for the generation of pAbs in relation to motifs present in huntingtin. (*a*) Antigens. The localization of the different peptides (ovals) and GST fusion proteins (bold lines) used for the immunization of rabbits are indicated. The numbers signify the names of the different sera. (*b*) Motifs. The putative nuclear localization signals for human huntingtin have been indicated (PSORT II; http://psort.nibb.ac.jp:8800/). Pat4 motifs are present at residue positions 89 (RPKK) and 2662 (RKHR); pat7 motifs are present at positions 86 (PLHRPKK) and 1182 (PIRRKGK). In addition, potential cleavage sites for apopain have been indicated (SWISS-PROT: P42858; http://expasy.hcuge.ch/). Potential cleavage sites are present at residue positions 513–514, 530–531, 552–553 and 589–590. N-terminal cleavage products of huntingtin with an expanded repeat might accumulate preferentially in HD brain (Davies *et al*. 1997; DiFiglia *et al*. 1997; Maat-Schieman *et al*. 1999).

patients has a crucial role in the molecular pathogenesis of this neurodegenerative disease is strengthened by the fact that transgenic mice that express only the first exon of the HD gene with an extended repeat of around 140 units display a severe neurological disorder with a lethal outcome (Mangiarini *et al*. 1996). In the brain of these mice, abnormal protein aggregates have been detected in the nuclei of neurons called neuronal intranuclear inclusions (NIIs) (Davies *et al*. 1997). The sensitivity of neuronal cells to this type of mutation is also suggested by the fact that at least seven other neurodegenerative disorders are associated with an expansion of polyglutamine-encoding CAG repeats. Furthermore, similar NIIs have been found in various brain regions of HD patients (DiFiglia *et al*. 1997) and of patients with spinocerebellar ataxia type 3, another polyglutamine repeat disorder (Paulson *et al*. 1997).

Nevertheless, these neurodegenerative diseases affect different parts of the brain, which suggests that other parts of the mutated proteins also contribute to the observed specific pathologies. Furthermore, in the transgenic mice that express only the first exon of the HD gene with the extended repeat, the specific cell death of neuronal cells in the striatum that is characteristic of HD is not observed. Accordingly, both the extended glutamine repeat and other domains of huntingtin might contribute to HD. Therefore, studies of the function of the glutamine-repeat region and an analysis of the function of the other segments of huntingtin are both required for a full understanding of HD pathology. Furthermore, detailed studies on the subcellular localization of this protein under various conditions might provide useful information. Essential tools for such studies include the availability of a well-characterized set of antibodies raised against various parts of huntingtin.

Here we describe the characterization of a panel of rabbit polyclonal antibodies raised against different regions of huntingtin. Purified polyclonal antibodies were used in immunofluorescence and in biochemical subcellular fractionation experiments with cells of neuronal origin. In addition, we studied the subcellular localization of huntingtin during neuronal differentiation *in vitro* and in HD autopsy brain.

2. MATERIALS AND METHODS

(a) *Generation of fusion proteins and antibodies*

Different huntingtin–glutathione *S*-transferase (huntingtin–GST) fusion genes were inserted into derivatives (pRP261, 265 and 269) of the pGEX vectors (Smith & Johnson 1988). GST fusion proteins were purified by using standard procedures and were subsequently used for the immunization of rabbits. Huntingtin–GST fusion proteins encompassing amino acid residues 596–1030, 852–1193 and 1929–2421 gave rise to serum

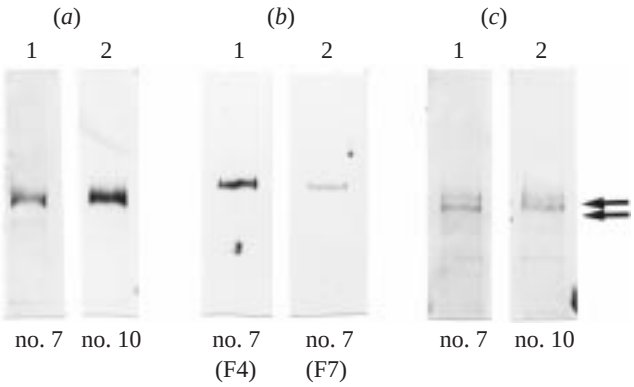

Figure 2. Western analysis with the use of pAbs no. 7 and no. 10 raised against a peptide encompassing the first 19 residues of huntingtin. (*a*) Analysis of crude sera. The results of Western experiments with enhanced chemiluminescence detection by using extracts prepared from human VH10 cells are shown for pAbs no. 7 and no. 10. (*b*) Affinity purification of pAb no. 10. Antibodies present in the serum were bound to a peptide column; eluted fractions were tested in Western blotting experiments with enhanced chemiluminescence detection with the use of extracts prepared from VH10 cells. F4 and F7 indicate fractions obtained after elution. (*c*) Detection of huntingtin with an expanded repeat. The results of Western blotting experiments with enhanced chemiluminescence detection with the use of extracts prepared from a HD patient (15–50-glutamine repeat) are shown for pAbs no. 7 and no. 10. Arrows indicate the position of full-length normal and mutated huntingtins.

97, serum 98 and serum 93, respectively. Synthetic peptides used for immunization were coupled to carrier proteins (bovine serum albumin (BSA), lysozyme or thyroglobulin) by using standard procedures (Harlow & Lane (1988), pp. 313–315). The distinct peptides used for this study contained residues 1–19 (sera 7, 10, 1859 and 1862), residues 11–19 (sera 2 and 5), residues 701–744 (sera 1359 and 1495) and residues 3114–3141 (sera 1356 and 1358). Rabbits were injected and bled monthly. Sera were tested with ELISA and Western blotting experiments.

(b) *Affinity purification of antibodies raised against peptides*

Antibodies no. 7 and no. 10 directed against the first 19 residues of huntingtin were affinity purified with an affinity column in accordance with the procedure supplied by the manufacturer (Pierce). In brief, a total of 2 mg of peptide was coupled to a column matrix and subsequently the sera were applied. After washing the column, bound antibodies were eluted with 0.1 M glycine, pH 2.5, and the collected fractions were immediately neutralized with 1 M Tris–HCl, pH 9.5. The purified antibodies were tested in Western blotting experiments.

(c) *Differentiation of neuroblastoma cells* in vitro

All cell lines were propagated as described (De Rooij *et al.* 1996). Mouse neuroblastoma cells (N1E-115) were grown under standard conditions in minimal essential medium supplemented with 2% (v/v) foetal calf serum (Life Technologies). Differentiation was induced by adding dimethyl sulphoxide to a final concentration of 1% (v/v). The subcellular localization of huntingtin was analysed in immunofluorescence experiments (days 0, 3 and 6 after the induction of differentiation).

(d) *Immunofluorescence, subcellular fractionation and Western blotting experiments*

Immunofluorescence, subcellular fractionation and Western blotting experiments were performed as described (De Rooij *et al.* 1996). Gel were scanned and analysed with the use of Gelworks (Ultra Violet Products, NonLinear Dynamics). Staining with 4′,6-diamidino-2-phenylindole (DAPI) was performed by the addition of this compound to the mounting medium (1 $\mu g\,l^{-1}$). VectaShield (Vector) was used as an anti-fading agent and mounting medium.

(e) *Immunohistochemistry*

Immunolabelling was performed on 5 µm thick sections cut from tissue blocks fixed in 10% (v/v) formalin and embedded in paraffin wax. Before pretreatment by heating in citrate buffer (pH 6.0) for 20 min, all sections were preincubated in 0.3% (v/v) H_2O_2 to block endogenous peroxidase. The primary antiserum no. 7 was incubated on the section overnight at room temperature. Next the section was incubated with biotinylated pig anti-rabbit immunoglobulin (Dako) for 30 min, followed by peroxidase-conjugated streptavidin (Dako) for 30 min. Peroxidase activity was detected with 3,3′-diaminobenzidine as the chromagen. Controls were obtained by omission of the primary antisera.

3. RESULTS

(a) *Generation and characterization of antibodies*

Rabbit polyclonal antibodies (pAbs) were generated against various regions of huntingtin by using either synthetic peptides or GST–huntingtin fusion proteins spread over the total huntingtin sequence (figure 1*a*). A total of 11 pAbs were obtained, raised against seven different antigens. Huntingtin can be cleaved by the pro-apoptotic protease apopain, generating an N-terminal fragment (Goldberg *et al.* 1996; see also figure 1*b*). In principle, only the pAbs raised against peptides present in the N-terminus can detect N-terminal cleavage products (i.e. nos 2, 5, 7, 10, 1859 and 1862; see also figure 1*a*). In figure 1*b* the position of amino acid sequences conforming to nuclear localization signals are also indicated.

All generated antisera were tested for reaction against huntingtin by using either ELISA (anti-peptide antibodies) or Western blots (anti-GST fusion proteins). All sera showed high reactivity against the antigens used for immunization. Subsequently we tested the capacity of the pAbs to recognize huntingtin in cell extracts with the use of Western analysis. A representative experiment is shown in figure 2. Antisera no. 7 and no. 10 were raised against a peptide encompassing the first 19 residues of huntingtin (see figure 1*a*). Both antibodies could detect one major band in the 200–400 kDa region of the blot (figure 2*a*). The same antisera were also purified by affinity chromatography. Figure 2*b* shows the results of the analysis of various fractions containing purified pAb no. 7. The results for the purification of pAb no. 10 were similar to those for pAb no. 7 (results not shown). The specificity of the observed interaction was underscored by the detection of two bands, i.e. huntingtin with a normal repeat (15) and an extended repeat (50) in extracts prepared from patient-derived lymphoblasts (figure 2*c*). In addition, pAbs no. 7 and no. 10 could also detect a clear expression

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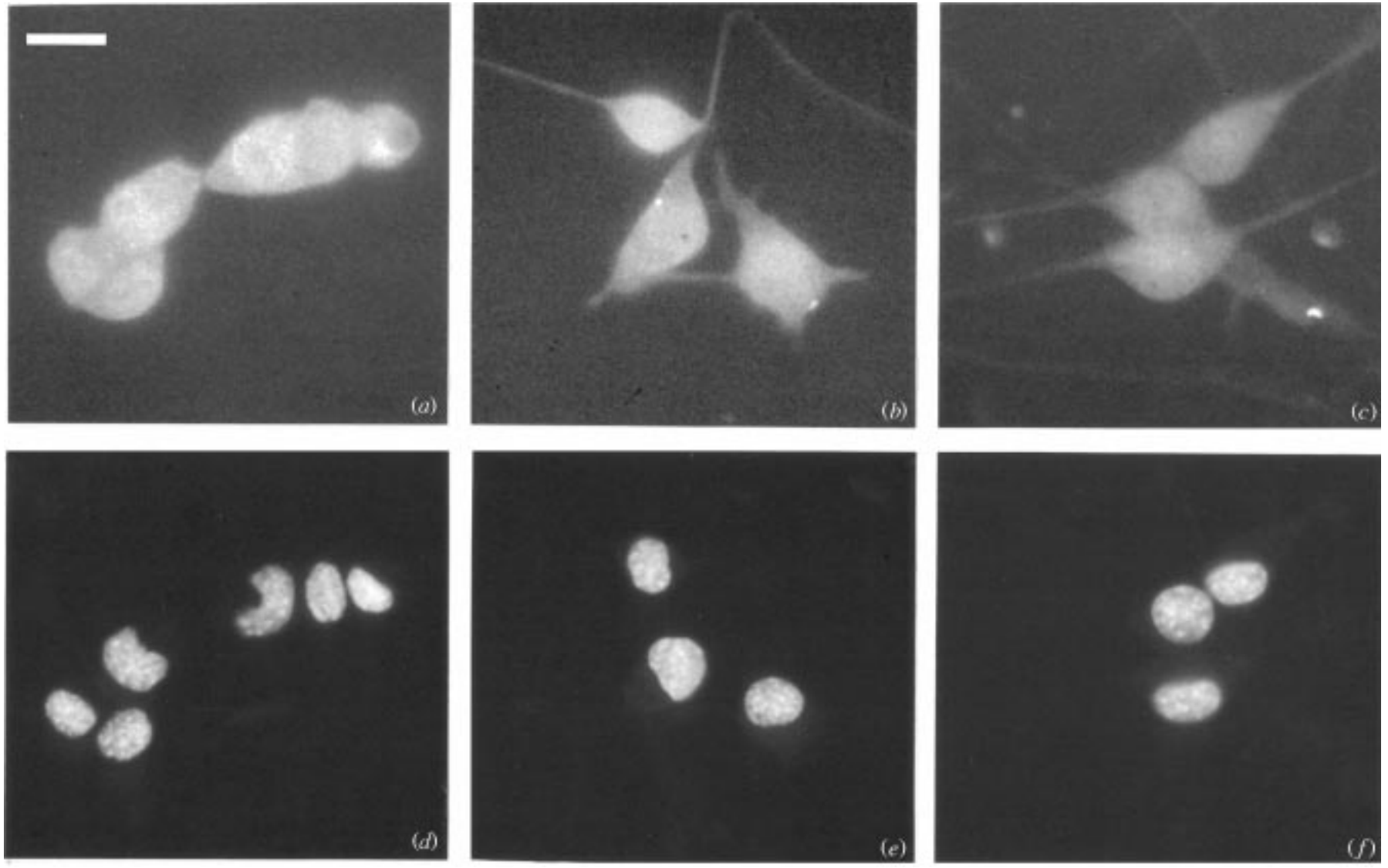

Figure 3. Subcellular localization of huntingtin in differentiating neuroblastoma N1E-115 cells. Immunofluorescence experiments with rabbit antibody pAb no. 10 are shown. (*a*) Undifferentiated cells; (*b*,*c*) cells after *in vitro* differentiation for 3 days (*b*) and 6 days (*c*). The lower panels (*d*–*f*) show the corresponding DAPI signals. No clear changes can be observed in the subcellular localization of huntingtin during neuronal differentiation. Scale bar, 20 μm.

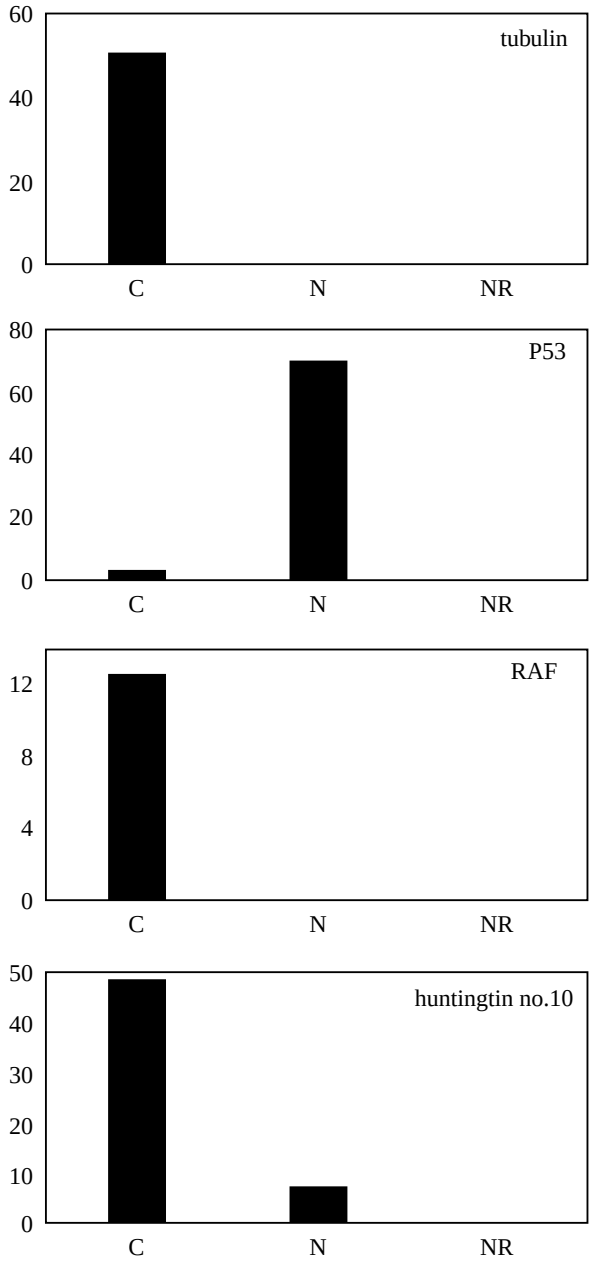

Figure 4. Subcellular fractionation studies. Undifferentiated mouse neuroblastoma N1E-115 cells were fractionated into cytoplasmic (C), nuclear (N) and nuclear remnant (NR) fractions, which were analysed in Western blotting experiments with pAb no. 10. The validity of the fractionation procedure followed was tested with antibodies against two different cytoplasmic proteins (tubulin and the kinase Raf) and against one nuclear protein (p53). After enhanced chemiluminescence detection of the signals, the films were scanned. A small amount of tubulin could be detected in the nuclear fractions, whereas the p53 protein and Raf were present exclusively in the nucleus and the cytoplasm, respectively. The scanning data have been corrected for trapping of a small amount of tubulin in the nuclear fraction. In agreement with the data of the immunofluorescence experiments, pAb no. 10 detects huntingtin both in the cytoplasm and the nucleus.

in *Caenorhabditis elegans* transgenic for exon 1 or exons 1–7 of the HD gene with $(CAG)_{17}$ and $(CAG)_{73}$ (results not shown). These results suggest that both pAbs efficiently recognize the N-terminus of the HD protein in the presence of relatively long repeat sizes and that these pAbs therefore constitute suitable reagents for the analysis of HD gene expression in human autopsy brain. All other antibodies were also capable of recognizing a protein in the expected size range, although with different affinities (results not shown).

(b) *Studies on the subcellular localization of huntingtin*

The pAbs were used to extend our studies on the subcellular localization of huntingtin. All (affinity purified) pAbs showed a comparable huntingtin signal in immunofluorescence experiments with human and mouse cells, including cells of neuronal origin. A representative immunofluorescence experiment is shown in figure 3. For this study the mouse neuroblastoma cell line N1E-115 was used. These cells constitute a suitable model system to study neuronal differentiation *in vitro* (see, for example, De Laat & Van der Saag 1982; Kimhi *et al*. 1976; Kranenburg *et al*. 1995). Cells were stimulated to differentiate by the addition of 1% (v/v) dimethyl sulphoxide, after which the cells flattened and neurites began to form from day 2 onwards. At day 6 the cells were fully differentiated. Immunofluorescence experiments with pAb no. 10 showed both a nuclear and cytoplasmic signal in each distinct stage of differentiation (figure 3*a–c*). On differentiation we observed no drastic changes in amount, appearance or subcellular localization of the staining.

To confirm the immunofluorescence results, a biochemical subcellular fractionation experiment was performed on undifferentiated N1E-115 cells. A procedure was followed that resulted in a cytoplasmic protein fraction, a nuclear protein fraction and nuclear remainders. The protein extracts were separated by SDS–PAGE, and Western blots were analysed with different huntingtin antibodies. The efficiency of the fractionation procedure was verified by analysing the distribution of the nuclear protein p53 and the cytoplasmic proteins Raf and tubulin. Films were scanned and the distribution of the proteins in the various fractions was calculated. As expected, p53 was detected exclusively in the nuclear fraction and Raf in the cytoplasm. Most tubulin was also detected in the cytoplasmic fraction, but a small amount was found in the nuclear fraction, indicating some carry-over of cytoplasmic proteins into the nuclear fraction during fractionation. The results obtained for tubulin were used to correct the scans of the other proteins, i.e. all calculated values for the nuclear fraction were lowered with a percentage equal to the percentage of tubulin found in the nuclear fraction (figure 4). With the use of pAb no. 10, after this correction *ca*. 85% of huntingtin is found in the cytoplasm and 10–15% is detected in the nucleus.

The pAbs were also used in an immunohistochemical study of autopsy brain of both juvenile-onset and adult-onset patients. In agreement with earlier studies, NIIs could be detected in the HD neostriatum and neocortex (Davies *et al*. 1997; DiFiglia *et al*. 1997). In figure 5, a strip of a HD cortex immunostained with antibody no. 7 is shown as an example. The NIIs could also be detected with pAb no. 10 but not with antibody no. 93, which reacted with the middle parts of huntingtin (results not shown).

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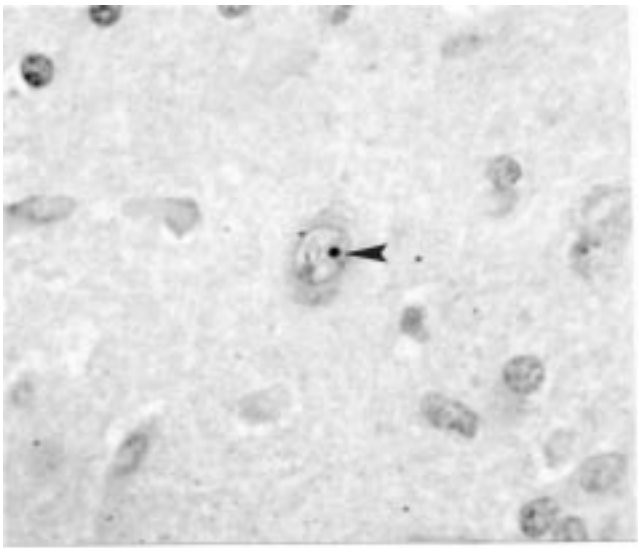

Figure 5. Immunohistochemical analysis of HD autopsy brain: a strip of HD neocortex showing neurons immunostained with pAb no. 7. Bound peroxidase-conjugated antibodies were revealed in a peroxidase reaction with 3,3′-diaminobenzidine as the chromagen. Controls were obtained by omission of the primary antiserum.

4. DISCUSSION

We have generated and characterized a panel of rabbit pAbs raised against distinct parts of human huntingtin, including against peptides of the extreme N-terminus and C-terminus. We have shown that all pAbs recognize human and mouse full-length huntingtin on Western blots and in immunofluorescence experiments. In this study the pAbs were used to extend our studies on the subcellular localization of huntingtin in cultured mammalian cells and in HD autopsy brain.

The attention was focused on the subcellular localization of huntingtin in mouse neuroblastoma N1E-115 cells, which are a well-established model for differentiation studies *in vitro* (see, for example, De Laat & Van der Saag 1982; Kimhi *et al*. 1976; Kranenburg *et al*. 1995). Most huntingtin was present in the cytoplasm and a minor fraction could be detected in the nucleus, both by biochemical subcellular fractionation and by immunofluorescence experiments. Furthermore, the nuclear signal could be detected with antibodies against the N-terminus, the middle parts and the C-terminus, suggesting that full-length huntingtin is present in the nucleus of cultured mammalian cells of neuronal origin. These results confirm and extend our earlier studies on the subcellular localization of huntingtin in several cell types (Hoogeveen *et al*. 1993; De Rooij *et al*. 1996).

We also analysed the amount and the subcellular distribution of huntingtin during neuronal differentiation *in vitro*. No significant changes were detected upon differentiation of N1E-115 cells into neurons. Studies with transgenic mice have indicated a critical function for huntingtin in neurogenesis (White *et al*. 1997). However, the role for huntingtin in neuronal development might not involve alterations in its subcellular localization.

In contrast, an altered subcellular localization of huntingtin, e.g. an increased accumulation of the N-terminal region of huntingtin in so-called NIIs in the neostriatum and neocortex, might contribute to the observed neurological defects in HD (Davies *et al*. 1997; DiFiglia *et al*. 1997). In an extension of these studies, NIIs were also detected in the HD-affected allocortex, but not in the pallidum, cerebellum and substantia nigra (Maat-Schieman *et al*. 1999). In all brain regions tested so far, only the N-terminal fragment of huntingtin seems to be present in the NIIs. The accumulation of full-length huntingtin with an expanded repeat, probably in other regions of the nucleus, has nevertheless also been reported (DiFiglia *et al*. 1997; Lunkes & Mandel 1998).

Various features of the HD protein might influence its subcellular localization and intranuclear distribution. Full-length huntingtin contains four putative nuclear localization signals (see figure 1*b*) and many phosphorylation sites (Hoogeveen *et al*. 1993; J. C. Dorsman and M. A. Smoor, unpublished data). It has been reported that phosphorylation in the vicinity of nuclear localization signals might alter the efficiency of import of proteins into the nucleus (see, for example, Xiao *et al*. 1997; Hubner *et al*. 1997). In particular, the cellular stress caused by the expression of huntingtin with an expanded repeat might affect the activities of many kinases and phosphatases, possibly including enzymes capable of modifying huntingtin. It is quite plausible that in cells expressing huntingtin with an expanded repeat, it is differently modified, leading to alterations in its subcellular distribution.

In addition, cleavage by the pro-apoptotic protease apopain seems to increase when huntingtin contains an expanded repeat (Goldberg *et al*. 1996) (see also figure 1*b*). For such N-terminal fragments the probability of a nuclear localization is higher than for the full-length protein, as calculated by the PSORTII program (http://psort.nibb.ac.jp:8800/). An increased nuclear import of huntingtin with an expanded glutamine repeat or N-terminal fragments thereof, coupled with the increased propensity for aggregation of the latter (see also Scherzinger *et al*. 1997) might well result in the formation of NIIs.

Whether the observed increase in accumulation of the N-terminal fragments of huntingtin with an expanded repeat in the NIIs and the concomitant functional consequences have a pivotal role in the aetiology of HD remains to be elucidated. It still remains possible that alterations in cytoplasmic and nuclear functions of (full-length) huntingtin contribute to the observed pathology, whereas its accumulation of NIIs is a parallel corollary of the alterations.

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