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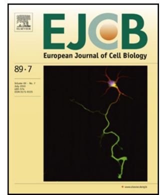

Complex interplay between the length and composition of the huntingtin-derived peptides modulates the intracellular behavior of the N-terminal fragments of mutant huntingtin

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ABSTRACT

Diverse subcellular localizations of the huntingtin-containing inclusion bodies are frequently suspected of reflecting crucial divisions between different cellular pathways contributing to the pathophysiology of Huntington's disease. Here, we use a panel of different N-terminal huntingtin fragments overexpressed in transfected neuronal and non-neuronal cells to demonstrate that it is the length of the N-terminal huntingtin fragments rather than a presence of any specific amino acid sequences that determines the ratio between the nuclear and cytoplasmic inclusion bodies. Importantly, the length of those fragments does also seem to strongly influence the folding of the aggregating huntingtin species, as indicated by the apparent differences in their accessibility for different antibodies directed against particular subdomains within the N-terminal part of huntingtin, although these differences do not correlate with the peptides' ability to efficiently aggregate within the cell nucleus. Furthermore, the relatively long huntingtin fragment containing 588 amino acids of the reference sequence shows intracellular behavior that is substantially different from that exhibited by its shorter counterparts (containing either 171, 120, 89 or 64 amino acids), as this rarely aggregating peptide is not only accumulating in cytoplasmic inclusions of slightly different morphology but is also most strongly affected by the FLAG-tagging procedure that unexpectedly induces (or enhances) autophagy-related processes. Together, our results reveal a significant heterogeneity of the huntingtin accumulation patterns that are observed at the cellular level. These patterns are not only strongly dependent on both the length and the amino acid composition of the N-terminal huntingtin peptides but also seem to engage different cellular mechanisms implicated in the pathogenesis of Huntington's disease, including the non-proteasomal degradation of potentially toxic huntingtin forms.

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Introduction

Intranuclear and cytoplasmic inclusions formed by short N-terminal huntingtin fragments encompassing a pathogenically elongated polyglutamine (polyQ) tract are a hallmark of Huntington's disease, a lethal neurodegenerative disorder characterized by selective degeneration of striatal neurons (Gil and Rego, 2008). Although the potential mechanisms linking this abnormal intracellular behavior of the huntingtin-derived peptides to the pathophysiology of Huntington's disease have been intensively studied over the past two decades, there are still more questions than answers when it comes to summarizing the current state of knowledge. For example, there is no general consensus regarding the most toxic form of huntingtin nor the biochemical or cellular pathway that is primarily responsible for the ultimate death of some specific groups of neurons.

While the initially assumed toxic function of the huntingtin-containing inclusion bodies is now commonly questioned, numerous studies have indicated that these large protein inclusions arise as a response to increased levels of some potentially toxic huntingtin-derived N-terminal fragments (Arrasate et al., 2004;Truant et al., 2008), which obviously allows us to treat such protein inclusions as excellent markers for the yet unknown processes directly involved in the pathophysiology of HD. Importantly, it has been recently noticed that the actual spectrum of all observed forms of both large inclusion bodies and small oligomeric

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aggregates of mutant huntingtin is much broader than previously suspected (for a review see Hatters (2012)), which urges for a much more thorough examination of all those relatively rare subtypes of huntingtin-containing aggregates that may potentially play a crucial role in the pathophysiology of HD and other polyglutamine disorders. In this context, it needs to be stressed that the nuclear aggregates formed by some relatively short huntingtin fragments have been frequently reported to be associated with an increased toxicity, hence they are commonly considered to be more strongly implicated in the pathogenesis of Huntington's disease when compared to their cytoplasmic counterparts (see, for example, Gutekunst et al., 1999, Peters et al., 1999, Schilling et al., 2004 and Yang et al., 2002).

The very first study systematically approaching the question of the relationship between the length and intracellular aggregation of huntingtin-derived peptides has established that while mutant fragments encompassing 224 or less N-terminal amino acids of the reference sequence are able to accumulate within nuclear inclusion bodies, this is not seen for fragments containing 427 or more amino acids (Hackam et al., 1998). Since then, the crucial role of the length of huntingtin fragments in subcellular distribution has been confirmed by numerous studies (for example Martindale et al. (1998), Cooper et al. (1998) and Lunkes et al. (2002)). However, it has remained possible that some specific amino acid sequences, either in the 224 – 427 aa region (Hackam et al., 1998) or within the 129 – 214 region (Lunkes et al., 2002), actively prevent the nuclear accumulation of huntingtin. Unfortunately, the interpretation of some of those results has been hampered by the unfortunate choice of antibodies used to detect some short huntingtin fragments (Hackam et al., 1998; Martindale et al., 1998) and by the fact that many different epitope tags were used to follow the subcellular distribution of particular huntingtin-derived fragments.

To investigate whether any specific amino acid sequence within the N-terminal part of huntingtin is either responsible for preventing intranuclear aggregation of some truncated variants of mutant huntingtin or contributes to any other well-known or relatively rare huntingtin accumulation pattern, we have analyzed the subcellular distribution of a series of huntingtin-derived peptides of different length in transfected neuronal and non-neuronal cells.

Material and methods

Expression plasmids

The plasmids encoding amino acids 1-588 of the full-length reference huntingtin sequence for both wild-type (Q17) and mutant (Q146) huntingtin, tagged N-terminally with the 3xFLAG epitope, were a gift from D.C. Rubinstein (Cambridge Institute for Medical Research, Cambridge, UK) (Luo et al., 2005). Site-directed mutagenesis system Transformer (Clontech) was used to modify this plasmid by deleting specific fragments of the gene and/or introducing new restriction sites facilitating the subsequent subcloning of different huntingtin fragments to the pRK5-KS (Becton Dickinson) or pCDNA3.1-Myc-His (Life Technologies) plasmids. The construction of the plasmid encoding the correct sequence of the 64-Q75 peptide tagged with epitopes Myc and His (including its different fusion variants) was described elsewhere (Bak and Milewski, 2010). The expression plasmid for HSP70 K71S was obtained from M. Żylicz (International institute of Molecular and Cellular Biology, Warsaw, Poland). The sequestosome (SQSTSM1) cDNA was purchased from OriGene (SC117750), subcloned into the pRK5-KS vector and modified (using site-directed mutagenesis) to introduce the sequence encoding epitope HA. All newly created expression plasmids were sequenced to verify their nucleotide sequence and then analyzed for protein expression using both western blot and immunofluorescence. The nucleotide sequences of all mutagenic and selection primers used to introduce the above changes are available upon request.

Cell cultures

Neuronal HT-22 cells, derived from a mouse hippocampus, were a gift from K. Domańska-Janik (Mossakowski Medical Research Centre, Warsaw, Poland), while the human HeLa cell line was purchased from Sigma-Aldrich (Cat. no 93021013). Both cell lines were cultured under standard cell culturing conditions (5% CO_2-balanced air at 37 °C) in DMEM (Gibco) supplemented with 10% fetal bovine serum (Gibco).

Transient transfections

Cells were seeded onto collagen-coated glass coverslips (for immunofluorescence analysis) or onto the plastic 6-well plates (for western blot analysis) and grown for 20 – 30 h until reaching 40 – 60% confluence. The HeLa cells were then transiently transfected with the FuGENE reagent (Promega), according to the manufacturer's instructions. In the case of the HT-22 cells, another transfection reagent (ExGen500, Fermentas) was applied. In all co-transfection experiments, a 1:1 DNA ratio was used for plasmids encoding different polypeptides. At 24 h after transfection, the cells were either lysed (for western blot) or fixed in 4% paraformaldehyde for 20 min at room temperature (for immunofluorescence).

Western blot analysis

To assess the protein levels in transiently transfected HeLa cells and to confirm the structural integrity of all overexpressed proteins, the transfected cells were lysed overnight in RIPA buffer (Sigma) and the total cell lysates were separated by SDS-PAGE (8%, 10% or 12%). The separated proteins were electrophoretically transferred to PVDF membranes (Amersham Pharmacia Biotech, Piscataway, NJ) and probed with monoclonal anti-Myc (Roche), anti-FLAG (SIGMA) or anti-huntingtin (MAB5492, MAB1574 and MAB5374) antibodies from Millipore. The ECL Plus system (Amersham Pharmacia Biotech) was used for the subsequent chemiluminescence detection step, according to manufacturers' instructions.

Fluorescent immunostaining

The immunostaining protocol for paraformaldehyde-fixed HeLa and HT-22 cells was essentially as previously described (Milewski et al., 2002). The monoclonal antibodies specified above plus the polyclonal anti-c-Myc antibody from Santa Cruz Biotechnology, the polyclonal anti-Flag antibody from Sigma-Aldrich and the polyclonal anti-LC3 antibody from Sigma-Aldrich were used as primary antibodies. FITC- or Cy3-conjugated anti-mouse and anti-rabbit immunoglobulins (Sigma-Aldrich) were used as secondary antibodies. The In Situ Cell Death Detection Kit (Roche) employing the TUNEL technology was used for the detection of apoptosis in some experiments.

Fluorescence microscopy and statistical analysis

The IX71 fluorescence microscope (Olympus) was used to analyze the protein distribution in transfected and immunostained cells. The presence of particular protein accumulations in at least 200 transfected cells was examined in duplicate in two separate experiments. Standard t-Student test was used to estimate the statistical significance (assumed at $p < 0.05$) of observed differences.

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