RESEARCH COMMUNICATION

Intracellular green fluorescent protein–polyalanine aggregates are associated with cell death

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Eight diseases, exemplified by Huntington's disease and spinocerebellar ataxia type 1, are caused by CAG-repeat expansion mutations. The CAG repeats are translated into expanded polyglutamine tracts, which are associated with deleterious novel functions. While these diseases are characterized by intraneuronal aggregate formation, it is unclear whether the aggregates cause disease. We have addressed this debate by generating intracellular aggregates with green fluorescent protein (GFP) fused to 19–37 alanines. No aggregates were seen in cells expressing native GFP or GFP fused to seven alanines. Aggregate-containing cells expressing GFP fused to 19–37 polyalanines show high rates of nuclear fragmentation compared with cells expressing the same constructs without aggregates, or cells expressing GFP fused to seven alanines. This suggests an association between aggregate formation and cell death.

Key words: Huntington's disease, oculopharyngeal muscular dystrophy, polyglutamine, trinucleotide.

INTRODUCTION

Eight neurodegenerative diseases have been described that are caused by CAG-trinucleotide repeat expansions coding for polyglutamine tracts. These include Huntington's disease (HD), spinocerebellar ataxias (SCAs) types-1, -2, -3, -6 and -7, spinobulbar muscular atrophy and dentatorubral–pallidoluysian atrophy (reviewed in [1]). Genetic and transgenic data suggest that these mutations cause disease by conferring deleterious novel function(s) on the relevant proteins. The diseases are likely to be directly related to the presence of the polyglutamine tracts, since cell dysfunction/death is caused by abnormally long polyglutamines alone, in abnormal genetic contexts, or in fragments of the various disease genes (reviewed in [1]).

It is not clear how expanded polyglutamine repeats cause cell death. A hallmark of many of these diseases, including HD [2], spinobulbar muscular atrophy [3], dentatorubral–pallidoluysian atrophy [4] and SCAs types-1 [5], -2 [6], -3 [7], -6 [8] and -7 [9], is the development of intracellular protein aggregates (inclusions) in the vulnerable neurons. A pathological role for inclusions is suggested by the correlation of inclusion counts in the cortex of HD patients with CAG repeat number, which reflects disease severity [10]. Inclusion formation precedes neurological dysfunction in some HD transgenic mice [11], and is associated with predisposition to cell death in *in vitro* models of HD [12–14], dentatorubral–pallidoluysian atrophy [15], spinobulbar muscular atrophy [16], SCA3 [7] and SCA6 [8]. Expanded polyglutamine-containing proteins form inclusions and cause death/dysfunction in both neuronal and non-neuronal cell lines (e.g. COS-7 [12–14]). Reduction of inclusions by overexpression of the heat-shock protein 40 HDJ-1 also decreases cell death [17].

A pathological role for inclusions in these diseases has been challenged by experiments reporting a dissociation between cell death and inclusion formation in primary cell cultures [18]. These findings were not straightforward, and might be compatible with a pathogenic role for huntingtin polymerization [1,19]. Klement et al. [20] deleted the self-association domain from an *SCA1* transgene with expanded repeats preventing inclusion formation, but this transgene caused an SCA-like phenotype in transgenic mice. These mice did not show the progressive disease seen in mice expressing *SCA1* transgenes with expanded repeats, including the self-association domain [21]. Since the latter mice develop inclusions, these aggregates may be necessary for disease progression. Recently, Cummings et al. [22] showed that a mutation in the E6-AP ubiquitin ligase reduces nuclear inclusion frequency while accelerating polyglutamine-induced pathology in SCA1 mice. Although these data suggest that large visible inclusions may not be required for cell death, the authors consider other possibilities that are consistent with a pathological role for inclusions. For instance, ubiquitination of ataxin-1 might not be E6-AP-dependent. The deletion of this enzyme might affect the turnover of other proteins [22], which, at abnormally high steady-state levels, might enhance the cellular sensitivity to the SCA1 mutation (or aggregates).

The above data have fuelled vigorous debate as to whether intracellular inclusions are deleterious, protective or epiphenomena [1,19]. One way of informing this debate is to make non-polyglutamine protein aggregates in cells and to test if these are associated with cell death. We fused polyalanine tracts of 7, 19, 25 and 37 residues to the C-terminus of enhanced green fluorescent protein (EGFP) (EGFP-C1; Clontech, Palo Alto, CA, U.S.A.), which we called EGFP–A_7, –A_{19}, –A_{25} and –A_{37} respectively. We used polyalanine tracts, since these are known to form β-sheets *in vitro* [23], and extended polyglutamine repeats may also form such structures *in vitro* [24] and *in vivo* [25]. Furthermore, expansions of 12 or more uninterrupted alanines in

Abbreviations used: (E)GFP, (enhanced) green fluorescent protein; HD, Huntington's disease; SCA, spinocerebellar ataxia; PABP2, polyadenine-binding protein 2; OPMD, oculopharyngeal muscular dystrophy; EM, electron microscopy.

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the polyadenine-binding protein 2 (PABP2), which is found in the nucleus, cause oculopharyngeal muscular dystrophy (OPMD) [26]. This disease is associated with the formation of nuclear inclusions, which probably contain aggregated PABP2 protein [26], although definitive data have not been published. We show here that extended polyalanine tracts form intracellular aggregates, and that these are associated with cell death.

MATERIALS AND METHODS

Expression constructs encoding EGFP with C-terminal polyalanine tracts of various sizes were synthesized by concatamerizing double-stranded oligonucleotides, and ligating these into the pEGFP-C1 mammalian expression vector (Clontech) using linkers. Complementary oligonucleotides, PAL1 (5′-GCCGCTGCCGCTGCTGCC-3′) and PAL2 (5′-GGCGGCAGCAGCGGCAGC-3′), encoding six consecutive alanines were phosphorylated using T4 polynucleotide kinase (New England Biolabs, Hitchin, Herts., U.K.), and annealed to generate double-stranded DNA linkers with complementary 3-bp 5′-overhangs. These were ligated overnight at 16 °C to allow concatamer formation, and products were then size-selected using low-melting-point agarose-gel electrophoresis. pEGFP-C1 was digested with *Bgl*II and *Hin*dIII, gel-purified, and then ligated to a linker made from the oligonucleotide pair LP1 (5′-GATCTGCAGCAGCC-3′)/LP2 (5′-GGCGGCTGCTGCA-3′). This was gel-purified, ligated with PAL1/PAL2 concatamers plus a linker made from the oligonucleotide pair and LP3 (5′-GCCGCTGCCGCTCA-3′)/LP4 (5′-AGCTTGAGCGGCAGC-3′), and transformed into *Escherichia coli* strain DH5α (Gibco BRL, Paisley, Renfrewshire, Scotland, U.K.). The size of the polyalanine stretch was determined by sequencing DNA from transformed colonies using EGFP-N and EGFP-C primers (Clontech). Tissue culture, transfection and fluorescence microscopy analyses of COS-7 cells were performed as described previously [14].

For electron microscopy (EM) studies, cells were fixed at 4 °C in 4 % (v/v) glutaraldehyde containing 0.01 % hydrogen peroxide and 3 mM $CaCl_2$ in 0.01 M Pipes buffer, pH 7.4. Cells were subsequently treated with osmium tetroxide and bulk-stained in uranyl acetate, dehydrated in an ascending series of ethanol solutions, and embedded in Spurr's resin. Thin sections were stained with uranyl acetate and lead citrate, and viewed in a Philips CM100 operated at 80 kV.

Pooled estimates for the proportions of cells with inclusions (or nuclear fragmentation) assessed in multiple experiments were calculated as odds ratios with 95 % confidence intervals [(percentage of cells expressing construct A with inclusions/percentage of cells expressing construct A without inclusions)/(% cells expressing construct B with inclusions/percentage of cells expressing construct B without inclusions)]. Odds ratios and *P* values were determined by unconditional logistical regression analysis, using the general log-linear analysis option of SPSS v6.1 software (SPSS Inc., Chicago, IL, U.S.A.).

RESULTS AND DISCUSSION

Expression constructs encoding EGFP with C-terminal polyalanine tracts of 7, 19, 25 and 37 were transfected into COS-7 cells, as described previously, and examined using fluorescence microscopy [14]. COS-7 cells were used because they are comparatively flat, and it is easy to see if their large nuclei are fragmented or not, which serves as an indicator of cell death. GFP fluorescence was distributed diffusely in cells expressing the pEGFP-C1 empty vector (results not shown) and the EGFP–A_7 constructs (Figure 1, upper left panel). In contrast, more than 20 % of cells expressing EGFP–A_{19}, –A_{25} and –A_{37} had mainly multiple inclusions 24 and 48 h after transfection (Figure 1, upper right panel). Inclusions appeared to be exclusively cytoplasmic. Cells had either multiple aggregates in normal-looking cytoplasm or multiple condensed aggregates. The GFP signal in these cells was sequestered to the aggregates. Cells were stained with 4,6-diamidino-2-phenylindole, and no nuclear aggregates were observed in cells analysed by fluorescent or confocal microscopy, where z-sections were viewed (results not shown). EM investigations of these inclusions revealed amorphous aggregates of material in the cytoplasm, varying in size from approx. 0.4–2 μm in diameter (Figure 1, lower panel). These contained distinguishable fibrils, approximating those seen in OPMD [26] and with recombinant aggregated polyalanine [23].

The solubility of the polyalanine aggregates was compared with polyglutamine aggregates formed by a mutant HD gene fragment (codons 8–57) fused to the C-terminus of EGFP-C1 (which we have called EGFP–HDQ_{74}, where 74 is the number of glutamines [14]). Post-transfection (48 h), live cells expressing polyalanine–EGFP fusion proteins were treated *in situ* with either 0.1 % (v/v) Triton X-100/0.1 % (w/v) SDS in 1 × PBS or 4 % (v/v) Triton X-100/4 % (w/v) SDS in 1 × PBS at room temperature, and examined using an inverted fluorescence microscope at regular intervals, as described by Housman and co-workers [27]. No fluorescent aggregates were detected with EGFP-C1 or EGFP–A_7 after treating cells with either 0.1 % Triton X-100/0.1 % SDS in 1 × PBS or 4 % Triton X-100/4 % SDS in 1 × PBS for 5 min (our first time point for measurement). In contrast, high-intensity GFP fluorescent aggregates were obtained from cells expressing EGFP–A_{25} at 5 and 10 min following treatment with 4 % Triton X-100/4 % SDS in 1 × PBS, but not at 25 min, and such aggregates were present up to 6 days following treatment with 0.1 % Triton X-100/0.1 % SDS in 1 × PBS (results not shown). EGFP–HDQ_{74} aggregates survived both treatments for up to 6 days, whereas EGFP–HDQ_{23} was solubilized after 5 min in both treatments. Preliminary data suggest that the formation of polyalanine aggregates is both time- and repeat-length-dependent, as we and others have observed for polyglutamine constructs [14] (results not shown).

COS-7 cells expressing EGFP–A_{25} were 2.11 times more likely to have fragmented nuclei than normal nuclei, compared with cells expressing EGFP–A_7 (three experiments; odds ratio of 2.11; 95 % confidence interval of 1.6–2.74; $P < 0.0001$) (Figure 2). Similarly, cells expressing 19 or 37 alanines also were more prone to death compared with cells expressing seven alanines (Figure 2). COS-7 cells expressing native pEGFP-C1 without added polyalanines showed diffuse fluorescence, and had levels of nuclear fragmentation similar to EGFP–A_7 (in a separate experiment, we found nuclear fragmentation in 17 % and 19 % of cells expressing native pEGFP-C1 compared with 19 % and 23 % in cells expressing EGFP–A_7). Nuclear fragmentation in cells expressing EGFP–A_{19}, –A_{25} and –A_{37} was strongly associated with aggregate formation. In all cases, cells expressing expanded polyalanine tracts with no aggregates had similar rates of nuclear fragmentation compared with cells expressing EGFP–A_7 (where the range of fragmented/normal nuclei was 25–28 % in three experiments). In the three experiments where we studied cells with EGFP–A_{25}, cells with aggregates were 14 times more likely to have fragmented nuclei than normal ones, compared with cells without aggregates (odds ratio of 14.3; 95 % confidence interval of 9.0–22.6; $P < 0.00001$). Data from a single representative experiment where we tested all of the constructs are shown in

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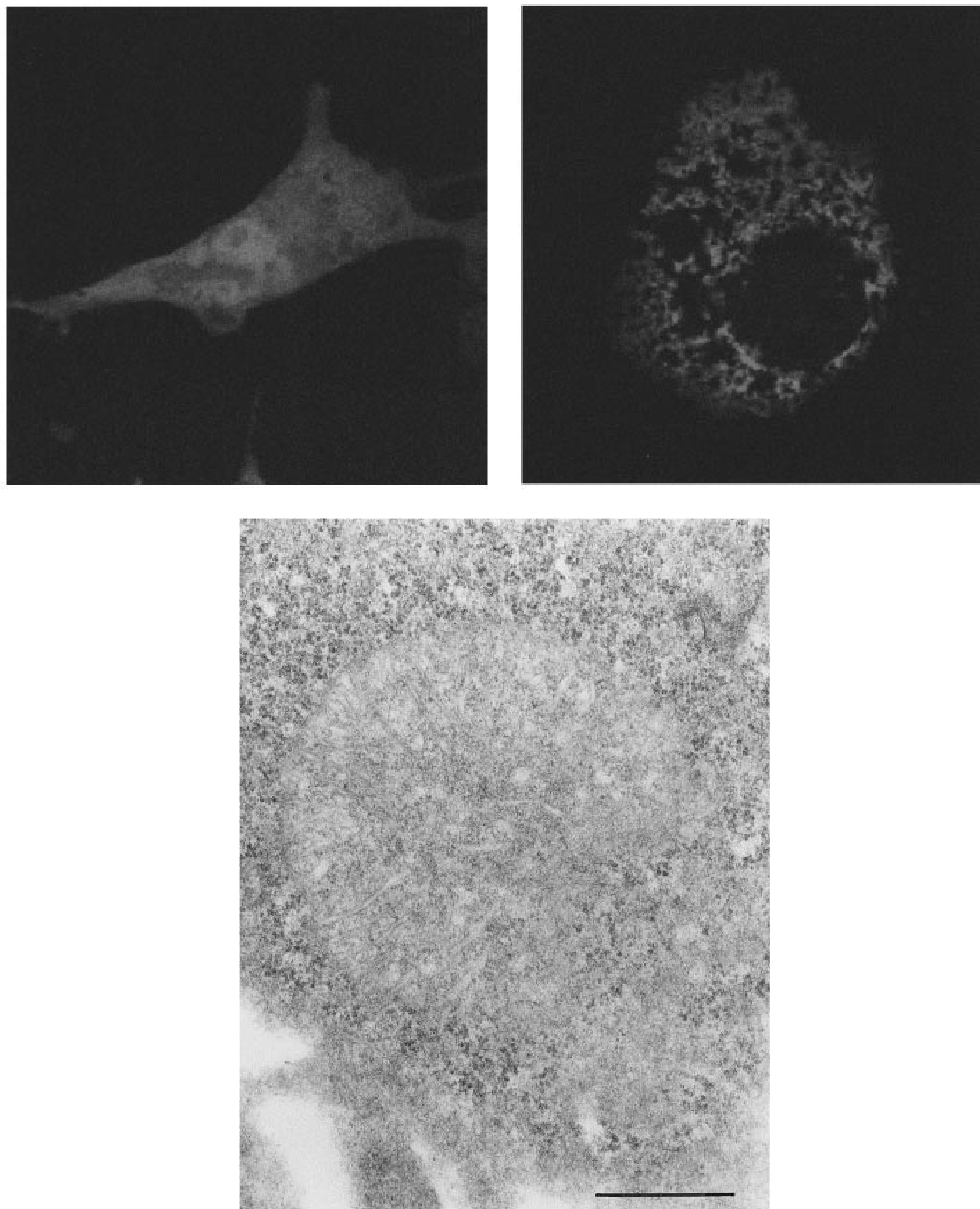

Figure 1 Polyalanine inclusions: fluorescent microscopy images of a COS-7 cell expressing EGFP–A_7 (upper left) or EGFP–A_{25} (upper right), and an electron micrograph of an aggregate (lower)

The aggregate is the more electron-lucent structure surrounded by electron-dense polyribosomes. The scale bar in the lower panel represents 500 nm.

Figure 2. Most of the inclusion-containing cells with fragmented nuclei appeared shrunken, with an irregular outline (results not shown). We have previously shown a similar association of cell death and aggregate formation in COS-7 cells expressing expanded polyglutamine tracts: EGFP–HDQ_{74}-expressing cells with inclusions showed significantly more nuclear fragmentation at 24, 48 and 72 h post-transfection compared with either EGFP–HDQ_{23}-expressing cells or EGFP–HDQ_{74}-expressing cells without inclusions, which had similar death rates [14].

Various authors have proposed that the formation of aggregates in polyglutamine diseases might be inherently pathogenic, protective or a secondary epiphenomenon [18–22]. Cells expressing either expanded polyglutamine or polyglutamine have abnormally high cell death in aggregate-containing cells com-

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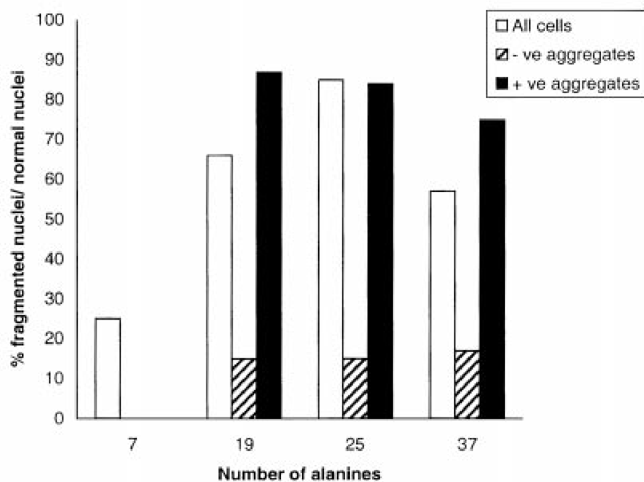

Figure 2 Nuclear fragmentation in COS-7 cells expressing GFP–A_7, –A_{19}, –A_{25} and –A_{37}

Data from an experiment where all of the mutant constructs were compared with the wild-type construct are shown. The proportion of cells with aggregates (+ve aggregates) that have nuclear fragmentation is also compared with that in cells without aggregates (−ve aggregates). At least 100 cells were counted per slide.

pared with cells without aggregates. It is thus unlikely that both polyalanine and polyglutamine expansions cause cell death in a manner unrelated to aggregate formation, and that the inclusions are a secondary epiphenomenon related to induction of death pathways. It is also unlikely that aggregates confer protection, and that these proteins are both toxic as soluble monomers, since one might expect that polyalanine and polyglutamine expansions would interact with a different subset of proteins, since glutamine is polar and alanine is non-polar. This might be a factor determining the neuronal specificity of polyglutamine expansions and the skeletal-muscle-specificity of OPMD. Our data cannot exclude the possibilities that small aggregates (invisible using light microscopy) might be more toxic than the larger aggregates that they may ultimately form, and that the cells with large aggregates might have been exposed to the deleterious conditions for a longer period. In practice, it may be difficult to test this hypothesis.

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