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Caspase 8 mediated apoptotic cell death induced by β-sheet forming polyalanine peptides

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Abstract Expansion of a polyalanine stretch from 10 to 12–17 residues in the N-terminus of the protein PABP2 has been implicated in the genetically acquired disease oculopharyngeal muscular dystrophy, characterized by nuclear protein deposits. Here we report a correlation between the structural properties and cell toxicity of two peptides mimicking the N-terminal domain of PABP2: one containing seven and the other 11 uninterrupted alanine residues. Consistent with earlier observations, the longer peptide (11-ala) was found to adopt β-sheet structure while the shorter one (7-ala) formed α-helix over a wide range of concentrations (∼20–500 μM). We observed that treatment with 11-ala resulted in significantly enhanced death of Chinese hamster V79 cells, compared to the effect of treatment with 7-ala, via the cytochrome *c* mediated apoptotic pathway. Increases in caspase 8 and caspase 3 activity were also observed in human cells (K562) treated with 11-ala. These results indicate that the toxicity of pathogenic peptides is directly linked to their β-sheet structure and also support recent observations that small oligomeric species of peptides and proteins are the key toxic elements in causing protein aggregation diseases.

Key words: Aggregation; Oculopharyngeal muscular dystrophy; Polyalanine; Circular dichroism; Beta-sheet; Apoptosis

1. Introduction

Expansion of the repeating trinucleotide sequences CAG and GCG, causing expansion of uninterrupted sequences of glutamine and alanine, respectively, in proteins has been identified as the causative mutations in a large number of inherited diseases [1–8]. An essential feature of polyglutamine expansion diseases is the presence of proteinaceous deposits in the neuronal cells of patients, leading to the term 'protein deposition disease' being coined for them [1]. Among the alanine expansion diseases, however, oculopharyngeal muscular dystrophy (OPMD) is the only one to exhibit the characteristic nuclear protein aggregation and filamentous nuclear inclusions in muscle fibers [9,10]. The autosomal dominant form of OPMD is caused by short expansions of a $(GCG)_6$ repeat to $(GCG)_{8-13}$ in the PABP2 gene, which results in the expansion of a polyalanine stretch from 10 to 12–17 alanines in the N-terminus of the protein [4]. Mutated PABP2 is able to induce nuclear protein aggregation and to lead to nuclear fragmentation [10].

A number of recent studies have demonstrated that aggregates of polypeptide tracts or fragments of protein implicated in protein misfolding diseases are directly toxic to cells. For instance, high levels of intracellular aggregates and nuclear fragmentation were observed in cells expressing green fluorescent protein (GFP) fused to 19–37 alanines, when compared to cells expressing GFP alone or GFP fused to seven alanines [11]. On the other hand, species formed early in the aggregation of non-disease associated proteins, such as the SH3 domain from bovine phosphatidylinositol 3′-kinase and the amino-terminal domain of the *Escherichia coli* HypF protein, have also been shown to be inherently highly cytotoxic [12]. Such results point to the need for exploring the role of other factors, e.g. protein sequence and conformation, in protein aggregation that may have a bearing on cell viability and thus would be relevant for the diseases.

One such factor is the length of the polypeptide tracts implicated in the disease. Not much is known about the relation between the lengths of polyalanine sequences and their pathological properties. Since expansions of 12 or more uninterrupted alanines in PABP2 are known to cause OPMD, we decided to look at peptides associated with this disease in the transition region between the monomeric and aggregated species and their concomitant effect, if any, on cells in culture.

We report here a correlation between the structural tendencies and the effect on viability of cells in culture of two peptides mimicking the N-terminal polyalanine segment of PABP2: one containing seven and the other 11 successive alanine residues. In addition to the N-terminal sequence of PABP2 (polyalanines preceded by a methionine and followed by a glycine), each contained a lysine at the N-terminus (to enable solution studies) and a tyrosine at the C-terminus to enable peptide concentrations to be determined spectrophotometrically. The sequences used were Ac-Lys-Met-$(Ala)_7$-Gly-Tyr (7-ala) and Ac-Lys-Met-$(Ala)_{11}$-Gly-Tyr (11-ala).

Polyalanine tracts containing >10 alanines have previously been shown to form β-pleated sheet complexes in solution, with $>50\%$ complex formation occurring for peptides containing ≥ 12 alanines [13]. Using circular dichroism (CD) spectroscopy we have verified that the two peptides used in this study adopt distinctly different secondary structures in solution. While the shorter peptide (7-ala) adopted exclusively α-helical structure, the longer one (11-ala) formed β-sheet and favored intermolecular association with increasing concentra-

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tion. The important new result found in this study was that 11-ala induced significantly higher levels of apoptosis compared to 7-ala when both were presented to Chinese hamster V79 cells in culture. To our knowledge, this is the first report of cellular toxicity of conformation specific (β-structure forming) oligomeric polyalanine peptides, raising the possibility that such peptides may act as useful model systems for elucidating the biochemical pathways of a class of protein misfolding diseases.

2. Materials and methods

2.1. Chemicals and antibodies

Hoechst 33258 dye and RNase A were obtained from Sigma Chemicals (USA) and proteinase K was from Life Technologies (USA). Nonidet P40 and nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) tablet were obtained from Boehringer Mannheim (Germany). Other molecular biology grade fine chemicals were procured locally. Anti-cytochrome *c* antibody was from PharMingen (Becton Dickinson, USA) and secondary antibody (anti-mouse) from Santa Cruz Biotechnology (USA).

2.2. Synthesis of oligopeptides

Peptide synthesis was performed on a LKB 4175 peptide synthesizer using the Fmoc polyamide active ester chemistry and solid phase peptide synthesis technique. They were cleaved with a solvent mixture containing 94% trifluoroacetic acid, 2% anisole, 2% ethanedithiol and 2% phenol at room temperature, precipitated by addition of diethyl ether, purified on a reversed phase C_{18} high performance liquid chromatography column and stored at −20°C in lyophilized powdered form. The purity of the peptides was confirmed by mass spectroscopic analysis on a MALDI-TOFSPEC 2E instrument (Micromass, UK).

2.3. Spectroscopic measurements

CD spectra were recorded on a Jasco J-720 spectropolarimeter using cylindrical quartz cuvettes of path length 1, 2 and 5 mm. Each spectrum represents the average of five successive scans performed at a scan speed of 20 nm/min. Appropriate baseline subtraction and noise reduction analysis were performed. At concentrations higher than 5×10^{-5} M the peptides were dissolved in a 60% acetonitrile–40% water mixture. For temperature dependent studies, a water jacketed cylindrical quartz cuvette of path length 2 mm was used in conjunction with a NESLAB RT-110 circulating water bath. Peptide concentrations were determined from tyrosine absorbance, using a molar extinction coefficient of 1280 M^{-1} cm^{-1} at 280 nm [14].

2.4. Electron microscopy (EM)

Samples of 11-ala for EM were prepared by incubation of a 300 μM (0.4 mg/ml) solution of the peptide in phosphate buffered saline (PBS, pH 7.4) at 65°C for 24 h. The samples were spread on carbon coated copper grids and stained with 2% (w/v) uranyl acetate in water. The air dried sample was visualized on a Hitachi H-600 TEM at 75 kV.

2.5. Cell culture

Chinese hamster V79 (male lung, fibroblast) cells were obtained from the National Institute of Virology, Pune, India. Cells were routinely grown as a monolayer in plastic Petri dishes using Eagle's minimal medium supplemented with dialyzed goat serum (complete medium) at 37°C in a humid atmosphere containing 5% CO_2 [15].

2.6. Treatment of cells with peptides

V79 cells were plated (10^6 per 100 mm Petri dish) in the complete medium and, after overnight incubation, treated with 20 μM final concentration of peptides (7-ala, 11-ala, bombesin and δ-melanocyte stimulating hormone) in 5.5 ml complete medium. Cells were then allowed to grow for another 36 h. At the end of the treatment the medium was removed, washed with PBS, trypsinized and suspended in PBS. Cells were counted under the microscope on a hemocytometer. Cells grown without addition of peptides were used as control. Growth inhibition by treatment with the peptides was calculated by dividing the number of viable cells obtained after treatment with peptides by the number of such cells obtained in untreated control. Cells were then used for detection of nuclear fragmentation, nucleosomal ladder formation or cytoplasmic cytochrome *c* release by Western blot as described below. Each experiment was repeated two to four times.

2.7. Analysis of nuclear and DNA fragmentation

Cells collected as above were washed with PBS twice, re-suspended in 70% ethanol (10^5 cells/ml) and kept at 4°C for 1 h for fixation. They were then stained with 1 mM Hoechst in PBS in the dark at room temperature for 5 min and observed under a fluorescence microscope (Olympus BX60 with appropriate attachments, Japan). About 200–500 cells were counted. Cells with intact nuclear morphology (normal) and fragmented nuclei (apoptotic cells) were determined and the fraction of cells with apoptotic nuclei was calculated.

DNA fragmentation assay was performed using a method incorporating some modifications of a standard one [16]. In brief, exponentially growing cells were treated with 20 μM peptides for 36 h as described above, after which they were harvested by trypsinization, washed with PBS and collected by centrifugation. The cell pellets were lysed in lysis buffer (50 mM Tris, 20 mM EDTA, 1% Nonidet P40) for 10 min at room temperature. The lysate was centrifuged at 2800 rpm and the supernatants collected. This process was repeated three times. The supernatant, after addition of sodium dodecyl sulfate (SDS, final concentration 1%), was treated with RNase A (final concentration 0.1 mg/ml) overnight at 56°C, followed by proteinase K (final concentration 0.1 mg/ml) for 6 h at 56°C. The lysate, after addition of a half volume of 10 M ammonium acetate, was treated with 2.5 volumes of absolute ethanol overnight at −20°C. The DNA was then precipitated by centrifugation at 12 000 rpm at 4°C for 15 min. After drying, the pellet was dissolved into sterile water and mixed with a gel loading dye (0.25% bromophenol blue, 40% sucrose), separated on 1.5% agarose gel and visualized under UV illumination after staining with ethidium bromide (0.5 μg/ml). The gel was photographed. Untreated cells were processed in the same way and used as control.

2.8. Western blotting

Chinese hamster V79 cells treated with peptides for 24 h and untreated control cells were harvested, washed twice with PBS and cytoplasmic extracts were prepared using standard methods [17]. Protein estimation was done according to Lowry's method: 20 μg protein was resolved on 15% polyacrylamide gel (acrylamide:bis-acrylamide 19:1) containing 0.1% SDS, transferred to nitrocellulose paper (Hybond ECL, Amersham Life Science, UK) and treated with blocking solution (5% bovine serum albumin in 25 mM Tris, 0.15 M NaCl, TBS) for 2 h at room temperature with constant shaking in an orbital shaker. Anti-cytochrome *c* (1:2000 dilution) was used to react overnight with separated proteins on the membrane in TBS containing 0.05% Tween 20 at 4°C. The blot was washed four times with 5.0 ml TBS containing 0.05% Tween 20, followed by treatment with alkaline phosphatase conjugated anti-mouse immunoglobulin G (at the same dilution as that of the primary antibody in TBS) for 2.5 h at room temperature. After washing the membrane four times with buffer and treating it with NBT/BCIP (substrate for alkaline phosphatase) to visualize the proteins, the blot was scanned and the intensities recorded.

3. Results and discussion

Fig. 1 shows the far UV CD spectra of the peptides over an almost 50-fold range of concentrations. The strong positive band at 192 nm and the twin minima at 207 nm and 222 nm in Fig. 1a show that 7-ala exists primarily in the α-helical conformation over the whole range. The degree of helicity ($\theta_{222\,nm}$) shows little variation with concentration while decreasing linearly with increasing temperature (inset), as expected for a simple monomeric species. For 11-ala, the strong positive band below 200 nm and the much weaker negative band around 220 nm (Fig. 1b) are indicative of formation of a β-type structure. With increase of concentration, the intensity of the positive band increases markedly and its position shifts to the red (by about 5 nm overall), a feature also observed in polyglutamine peptides and attributed to widening of the

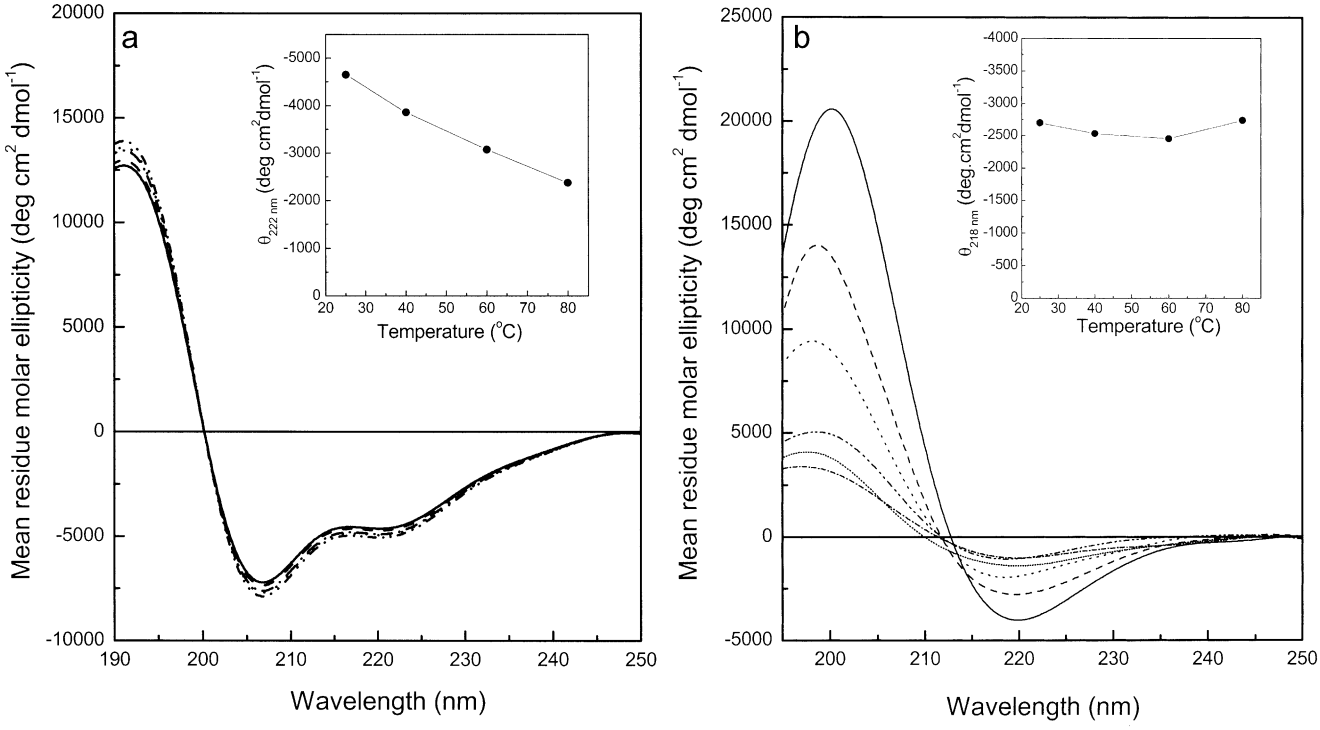

Fig. 1. Concentration dependent CD spectra of the peptides at 25°C. a: For 7-ala at concentrations 4.25×10^{-4} M (——), 2.12×10^{-4} M (– – –), 1.06×10^{-4} M (·····), 5.31×10^{-5} M (–··–··–) and 2.65×10^{-5} M (–·–·–). Inset shows temperature dependent ellipticity at 222 nm. b: For 11-ala at concentrations of 4.2×10^{-4} M (——), 2.8×10^{-4} M (– – –), 1.4×10^{-4} M (·····), 7.0×10^{-5} M (–··–··–), 3.5×10^{-5} M (.....) and 8.7×10^{-6} M (–·–·–). Inset shows the temperature dependent ellipticity at 218 nm.

β-sheets and/or formation of tightly linked and twisted *intermolecular* species [18]. The strong nature of the intermolecular association is also indicated by the thermal stability of $\theta_{218\ nm}$ (inset), which characterizes the β-sheet structure. These results verify the structural transition of polyalanine sequences of length >10 residues from random coil/α-helix to β-pleated sheets, as reported earlier by Blondelle et al. [13].

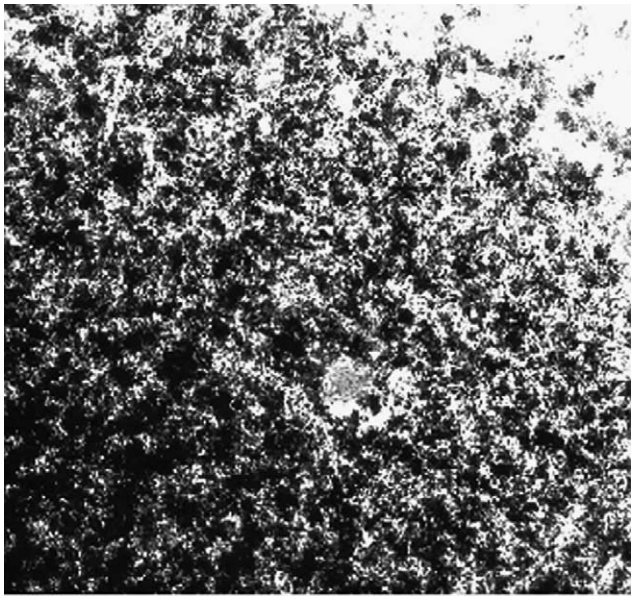

Fig. 2. Electron micrograph of negatively stained samples of 11-ala prepared by incubation at 65°C for 24 h. Magnification: 60 000×.

Fig. 2 shows a typical electron micrograph of the 'aged' 11-ala peptide, where amorphous clumps of average dimension 30–50 nm are visible. Polyalanine aggregates of similar size have been reported, though no amyloid-like fibrillar forms or networks were detected in the present case even after extended periods of ageing [13].

To explore the deleterious effects of the β-structure forming peptides, equal numbers of Chinese hamster V79 cells were treated with 20 μM of the peptides (7-ala, 11-ala) for 36 h. Cells treated with the 11-ala peptide showed a significant decrease in growth compared to untreated control. A small decrease in growth occurred for cells treated with 7-ala, while the number of untreated cells remained unchanged. Averaged results of three to five independent experiments indicated that treatment with 11-ala inhibited cell growth by 55%, whereas for the 7-ala treated cells this value was 18%. This difference was statistically significant ($P=0.03$).

The morphology of the nuclei was examined after staining with the DNA binding dye Hoechst 33258. Cells treated with 11-ala had a much higher number of nuclei with typical morphology for nuclear fragmentation. A composite picture is shown in Fig. 3a. The average number of untreated (control) cells that exhibited apoptosis was (9 ± 9)%. This value was (15 ± 8)% and (29 ± 9)% for 7-ala and 11-ala treated cells, respectively. The difference between the values for the untreated cells and the 7-ala treated cells was not statistically significant ($P=0.36$). However, this difference was statistically significant ($P=0.02$) for cells treated with the 11-ala peptide. The induced frequency of apoptotic cells for treatment with 7-ala and 11-ala peptides was significantly different ($P=0.002$), as shown in Fig. 3b. A comparison of the ability of different concentrations of 11-ala to induce apoptosis is

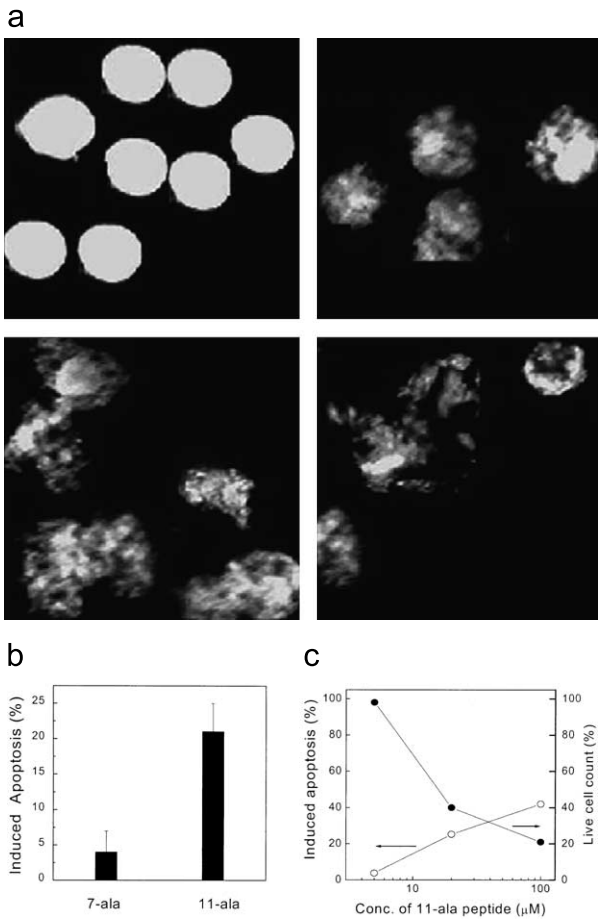

Fig. 3. Induced apoptosis as detected by nuclear morphology of Chinese hamster V79 cells stained with Hoechst 33258 under a fluorescence microscope. a: Typical examples of normal and apoptotic morphology. b: Bar diagram of induced apoptosis (determined by subtracting the frequencies obtained for untreated control cells) resulting from treatment by the two peptides. c: Comparison of different concentrations of the 11-ala peptide for their ability to induce apoptosis.

shown in Fig. 3c. An apoptotic level of 40% was reached for treatment with 100 μM of peptide.

We also investigated the ability of the peptides to induce the nucleosomal ladder, a hallmark of apoptosis. The result of a typical experiment is shown in Fig. 4a. It is evident that 11-ala could induce the nucleosomal ladder and the intensities of the ladders were higher than in the untreated control. 7-Ala treatment also resulted in a similar pattern as that of the control (data not shown). This differential result is similar to that observed with nuclear morphology described above.

In apoptosis, cytochrome *c* – which usually remains in the mitochondria – is released in the cytoplasm and in turn activates the caspase cascade leading to nucleosomal ladder formation. In the conditions of our experiments, untreated control cells also released a small amount of cytochrome *c* in the cytoplasm. The extent of cytochrome *c* release varies from one experiment to another. The result of a typical experiment is shown in Fig. 4b, in which the intensities of the bands reflect an eight-fold increase of cytochrome *c* release in cells treated with 11-ala, compared to untreated control.

To investigate the role of caspase 8 in the observed apoptosis induction by 11-ala, we treated the human cell line K562 with 20 μM peptide (final concentration) for 20 h. Similar to what we found with Chinese hamster V79 cells, such treatment inhibited the growth of cells, increased the release of cytochrome *c* and caspase 8-like activity. Caspase 8 and caspase 3 activities were detected fluorometrically using commercially available kits (Chemicon International, USA and PharMingen (Becton Dickinson), USA for caspase 8 and caspase 3, respectively) following methods recommended by the manufacturers. In a typical experiment we observed 45% cell growth inhibition and ~3-fold increase in cytochrome *c* release over the control (data not shown). In the caspase 8 assay, there was a ~10-fold increase in fluorescence over untreated control (from 37 to 390 in arbitrary units (au)). For the caspase 3 assay, a two-fold increase in fluorescence over untreated control was detected (83 to 163 au). This set of data indicates that treatment with 11-ala in conditions where they formed β-structures increased apoptosis of cells by the caspase 8 mediated pathway, together with cytochrome *c* release and caspase 3 activation. Although increased caspase 8 activity and apoptosis have been reported in cells expressing pathologically long polyglutamine sequences, this study is the first to report similar effects of polyalanine peptides in a conformation specific manner [19].

Polyglutamine stretches of length ≥37 in proteins have been implicated in a number of neurodegenerative diseases and are known to form aggregates as nuclear inclusion bodies and induce apoptosis [19]. However, the role of these aggregates in the molecular pathogenesis of the diseases is unclear. While formation of aggregates and nuclear inclusions has been shown to precede neurological abnormality in Huntington's disease model systems, observations dissociating aggregation formation from cell death have also been reported [20]. Treatment of cells with non-disease associated proteins in conditions favoring aggregate formation was found to induce toxicity, although the type of cell death (apoptosis or necrosis) in such cases was not determined [12,21]. Treatment of neuroblastoma cells with mutant and wild type α-synuclein that are capable of aggregate formation was found to induce nuclear fragmentation, an indication of apoptosis [22]. In both instances the toxic species formed highly organized amyloid-like fibrillar structures on ageing, although cytotoxicity in the first case was far more pronounced for the rapidly formed non-

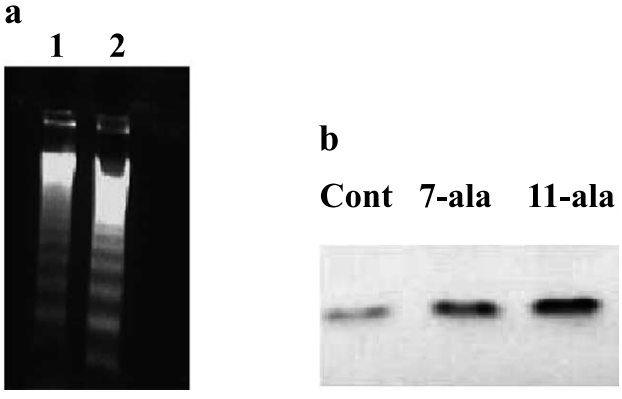

Fig. 4. Detection of apoptosis by nucleosomal ladder formation and cytochrome *c* release into the cytoplasm. a: DNA fragmentation into nucleosomal ladders: lane 1 represents the untreated control, while lane 2 represents cells treated with 11-ala. b: Cytochrome *c* release: lanes are labeled at the top as cont (untreated control), 7-ala and 11-ala (treated with 7-ala and 11-ala, respectively).

fibrillar aggregates than for the longer filamentous structures adopted by the proteins later during incubation. In our experiments demonstrating apoptosis induction by 11-ala, pathological behavior was not correlated with large scale amyloid-type aggregate formation. Although incubation of 11-ala did produce amorphous clumps, amyloid-like fibrillar forms were not detected even after extended ageing. This result lends credence to the recent hypothesis that small oligomers preceding fibril formation may be the primary toxic species in causing disease [12,21,23].

In summary, we have shown that a β-sheet forming peptide containing 11 successive alanine residues shows concentration dependent intermolecular association and is highly effective in inducing apoptosis, a trait not exhibited by a related peptide containing a stretch of seven alanines that adopts α-helical conformation over the same range of concentrations. That apoptosis was mediated through the mitochondrial pathway was indicated by the release of cytochrome *c* in the cytoplasm. At this point it is not clear whether or how these peptides enter the cells. It has recently been shown that polyglutamine aggregates can enter cells and induce apoptosis, possibly through the activation of caspase 8 or caspase 10 [24–26]. The detection of increased caspase 8-like activity induced by 11-ala suggests that this could also be the case here.

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