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A short Id2 protein fragment containing the nuclear export signal forms amyloid-like fibrils ☆

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Abstract

The negative regulator of DNA-binding/cell-differentiation Id2 is a small protein containing a central helix-loop-helix (HLH) motif and a C-terminal nuclear export signal (NES). Whereas the former is essential for Id2 dimerization and nuclear localization, the latter is responsible for the transport of Id2 from the nucleus to the cytoplasm. Whereas the isolated Id2 HLH motif is highly helical, large C-terminal Id2 fragments including the NES sequence are either unordered or aggregation-prone. To study the conformational properties of the isolated NES region, we synthesized the Id2 segment 103–124. The latter was insoluble in water and only temporarily soluble in water/alcohol mixtures, where it formed quickly precipitating β-sheets. Introduction of a positively charged N-terminal tail prevented aggressive precipitation and led to aggregates consisting of long fibrils that bound thioflavin T. These results show an interesting structural aspect of the Id2 NES region, which might be of significance for both protein folding and function.

Keywords: Nuclear export signal; Id2 protein; Fibrils; Circular dichroism; Electron microscopy; Peptide aggregation

The inhibitor of DNA-binding/cell-differentiation Id2 is one of the four members (Id1-4) of the Id family of helix-loop-helix (HLH) proteins which, contrarily to the related basic-HLH (bHLH) transcription factors, do not possess an N-terminal basic region for DNA-binding [1,2]. The Id HLH motif is essential for the formation of heterodimers with several bHLH factors, including the ubiquitously expressed E proteins and the tissue-specific myogenic-regulating factors: as the Id subunit lacks the basic region, these dimers are unable to bind the DNA and thus to activate DNA transcription [3]. Based on such protein–protein interaction mechanism, the Id proteins control many biological events like cell proliferation and differentiation, development [4], and tumorigenesis [5,6].

Whereas the HLH motif in all four Id proteins seems to have predominantly a structural function, the role of the N- and C-terminal regions is not yet completely understood: it is known that phosphorylation of Ser5 modulates the inhibitory activity of Id2 and Id3 [7–9], and that the N-terminal amino group of Id2 is essential for ubiquitination [10]. Moreover, the N-terminus of Id2 can induce apoptosis independently of the HLH dimerization motif [11]. Recently, Kurooka and Yokota have shown that the HLH region of Id2 is required for the protein nuclear localization, whereas residues 103–119 in the central part of the C-terminus are necessary for the localization of the protein in the cytoplasm. They also demonstrated that the isolated 17-residue long Id2 fragment displays nuclear export activity when conjugated to other proteins [12]. Indeed, the sequence 106–115 has been identified as the nuclear export

☆ *Abbreviations:* Ahx, 6-aminohexanoyl; CD, circular dichroism; DIPEA, *N*,*N*-diisopropylethylamine; DMF, *N*,*N*-dimethylformamide; EM, electron microscopy; Fmoc, 9-fluorenylmethoxycarbonyl; HBTU, *O*-(1-benzotriazolyl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HOBt, *N*-hydroxybenzotriazole; NMP, *N*-methylpyrrolidone; TFA, trifluoroacetic acid; TFE, 2,2,2-trifluoroethanol; ThT, thioflavin T.

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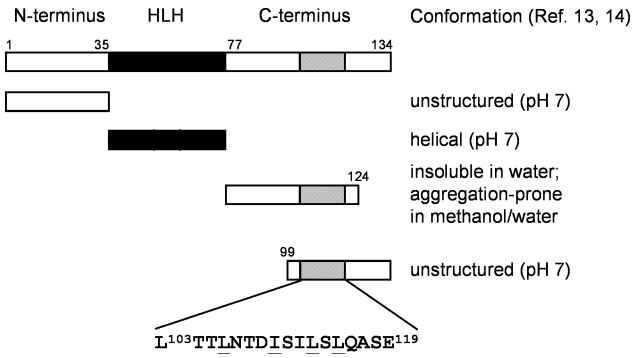

Fig. 1. Schematic representation of the full-length Id2 protein and of synthetic fragments reproducing the N-terminus, the HLH motif, and parts of the C-terminus. The gray bar represents the Id sequence 103–119 that is required for the Id2 nucleo-cytoplasmic transport. The key residues of the NES consensus are underlined.

signal (NES), in which Leu106, Ile110, Leu113, and Leu115 are the key residues.

We are interested in the conformation of the Id proteins and have shown that synthetic peptides reproducing the central HLH motifs are helical, whereas fragments derived from the N- and C-terminal domains have no preferred structure under physiological conditions (100 mM phosphate buffer, pH 7.3) [13]. In the case of Id2, the flanking region C-terminal to the HLH motif spans from residue 77 to residue 134; by examining the two fragments 99–134 and 77–124, we found that the former was soluble and unstructured in phosphate buffer, whereas the latter was insoluble under the same conditions (Fig. 1) [14]. The different behavior of these two peptides, which share the NES sequence but display different N- and C-termini, suggests that the properties of the NES segment can be modulated by the neighboring regions. However, as the isolated Id2 NES is functional, we were wondering whether it adopts a preferred conformation or is rather flexible. Thus, we have undertaken the present work to elucidate the structural features of short synthetic peptides based on the Id2 NES sequence. The results show that the Id2 NES region has high propensity to form β-sheets which aggressively precipitate in form of short fibrils combined with amorphous material.

Materials and methods

Peptide synthesis. The peptides used in this work are reported in Table 1. They were prepared by solid-phase methodology using the procedure described previously [14]. Briefly, double coupling (2 × 40 min) of the N^{α}-Fmoc-amino acids was accomplished by activation in situ with HBTU/HOBt/DIPEA. Fmoc cleavage was accomplished in DMF/NMP (80:20, v/v) with 40% piperidine for 3 min, followed by a second treatment with 20% piperidine for 10 min. The N-terminus was acetylated with acetic anhydride and DIPEA. The peptides were cleaved from the Rink amide 4-methylbenzhydrylamine resin and simultaneously deprotected by using the mixture TFA/water/triisopropylsilane (90:5:5 v/v). The molecular masses ($[M + H]^+$, Da) were determined by MALDI-TOF mass spectrometry. The retention times (t_R, min) were obtained from the analytical HPLC runs on a reverse-phase C18 column, with the binary system (A) 0.012% TFA in water and (B) 0.01% TFA in acetonitrile (gradient: 10–70% B over 40 min. UV detection at 220 nm).

CD spectroscopy. The CD spectra were recorded on a JASCO J710 spectropolarimeter, using a thermostatable quartz cell with a path length of 0.02 cm. For each CD spectrum 10 scans were accumulated with the following parameters: 1 nm step resolution and band width, 2 s response time, 20 nm/min scan speed, and 20 mdeg sensitivity. The CD spectrum of the solvent was subtracted from that of the peptide to eliminate interferences from the cell, solvent and optical equipment. The noise reduction was obtained by a Fourier transform filter with the program Origin (OriginLab Corporation, Northampton, MA, USA).

Electron microscopy. Formvar-/carbon-coated, glow-discharged copper grids were incubated with small volumes of the peptide suspensions (30 μl) for 5 min, blotted dry with a filter paper and air-dried for 10 min. Afterward, negative staining was performed with 2% aqueous phosphotungstic acid (pH 7.2) or 2% aqueous uranyl acetate (pH 4.1) by using the droplet technique. The samples were then air-dried for at least 30 min and visualized on a LEO912AB electron microscope (Zeiss, Oberkochen/Germany) operating at 80 kV, equipped with a bottom-mounted CCD-camera and with the EsiVision software (version 3.2, Soft Imaging systems, Muenster/Germany).

Turbidity assay. UV spectra of the freshly prepared peptide solutions at the concentration of 50 μM in methanol/water mixtures (50:50 or 20:80, v/v) were recorded in the region 280–500 nm at intervals of five up to 30 min over 260 min. The increment of the apparent absorbance at 320 nm, which is related to the turbidity of the solution, was then plotted against the time.

Thioflavin T assay. Aliquots of the 10-day peptide samples used for the EM experiments (25 μl) were diluted with 1 ml of ThT solution (5 μM in methanol and in mixtures of methanol/water 80:20 and 20:80). The final peptide concentration was 1.2 μM. The samples were pipetted vigorously and the fluorescence emission of ThT (λ_{ex}: 446 nm, λ_{em}: 484 nm) was measured on a Cary Eclipse spectrophotometer.

Results and discussion

The choice of studying the Id2 sequence 103–124 (peptide **1**, Table 1) instead of the shorter one 103–119 reported as the active Id2 NES by Kurooka and Yokota [12] has been dictated by synthetic reasons, as the longer fragment was found to be much more easily accessible than the shorter one [14]. However, despite its ease of synthesis, peptide **1** displayed the disadvantage of being insoluble in water. For this reason, the CD spectra of **1** were recorded in methanol or TFE and upon addition of up to 50% water (at higher water percentages there was instantaneous

Table 1
Synthetic peptides containing the NES region of Id2[a]

No.	Sequence	M_{calc} (Da)	M_{found} (Da)	t_R (min)
1	L103TTLNTDISILSLQASEFPSEL124	2432	2434	24.0
2	KKKX-L103TTLNTDISILSLQASEFPSEL124	2930	2931	21.5
3	L103TTLNTDISILSLQASEFPSEL124-XKKK	2930	2932	21.1

[a] The sequences are N-terminally acetylated and C-terminally amidated (X = Ahx).

peptide precipitation). In methanol, the NES sequence adopted a β-sheet structure, as indicated by the negative CD band at 214 nm and the positive one at 193 nm, with a crossover at 202 nm (Fig. 2A). Upon water addition, there was a significant decrease in the CD intensity, which should be attributable to the formation of oligomers stabilized by hydrophobic interactions. In contrast, a helix conformation was induced in TFE, which remained stable upon addition of up to 50% water (Fig. 2B).

In order to improve the water solubility of peptide **1** or at least to slow down its precipitation from alcohol/water, which would allow to perform experiments requiring long measurement times (e.g., 2D NMR), we decided to modify the sequence by increasing the number of positively charged residues, an approach that has been successful to stabilize amyloidogenic peptides in solution like PrP 174–195 [15]. As such modification should obviously not affect the conformation significantly, we chose the motif $(Lys)_3$–Ahx to be coupled to the N-terminal residue 103 (variant **2**) or to the C-terminal residue 124 (variant **3**) (Table 1). Beside three ammonium ions, this motif displays the Ahx unit that functions as a spacer between the basic moiety and the NES region, which should also reduce the risk of influencing the conformation of the latter. Although both analogs were still insoluble in water, they could be dissolved in methanol/water or TFE/water mixtures containing up to 80% water. Moreover, the results of the turbidity assay showed that the solution form of peptide **3** was much more stable than that of peptide **2** (Fig. 3). The effect of the N- or C-terminal modification of peptide **1** on its conformation was investigated by CD spectroscopy. In methanol, peptide **2** was characterized by a very intense β-sheet-like CD curve (Fig. 2C). As observed in the case of peptide **1**, water addition decreased the intensity of the CD spectra, thus suggesting the appearance of β-sheet aggregates stabilized by hydrophobic interactions. It is also plausible to assume that the β-strands were in an antiparallel arrangement, which would allow favorable electrostatic interactions involving the basic N-terminal lysine residues and the C-terminal glutamic acid residues (Glu119/123) (Fig. 4). Additionally, aromatic-cation interactions between Phe120 and the lysine side chains might also have a stabilizing effect [16]. Importantly, the CD spectrum of

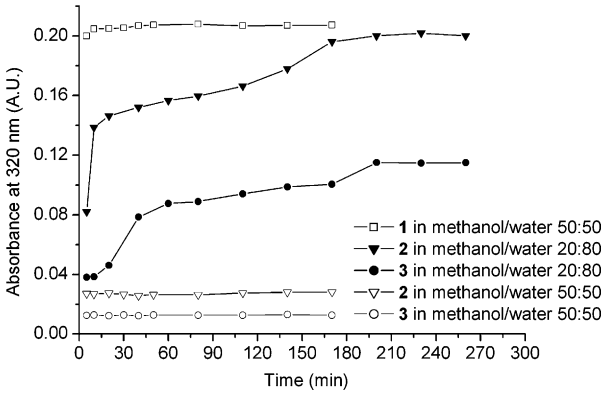

Fig. 3. Time-dependent appearance of turbidity in solutions of peptides **1**–**3** (50 μM in methanol/water mixtures).

Fig. 4. Proposed antiparallel alignment of β-strands of peptide **2**.

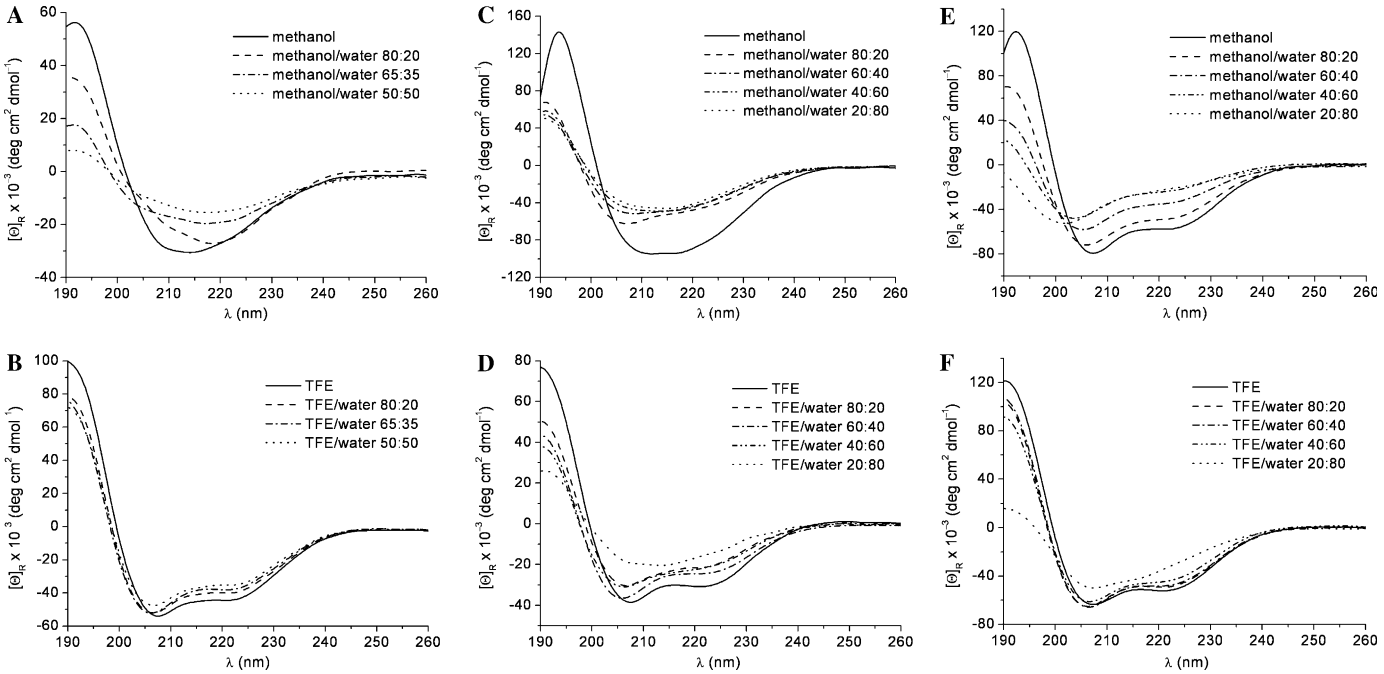

Fig. 2. CD spectra of peptides **1** (A,B), **2** (C,D), and **3** (E,F) (50 μM each) in methanol/water and TFE/water.

peptide **2** in methanol/water 40:60 or 20:80 was similar in shape and intensity to that of peptide **1** in methanol, indicating that analog **2** was structurally close to the native fragment 103–124, but it had the advantage of requiring smaller amounts of organic solvent to be dissolved and to remain in solution for a longer period. Also in TFE peptide **2** behaved like peptide **1**, building a α-helix that was stable upon addition of up to 60% water (Fig. 2D). At higher water percentage, peptide **2** underwent a conformational change from α-helix to β-sheet, which was not observed for peptide **1** (Fig. 2B), thus showing its strong tendency to adopt β-sheets even in a well-known α-helix-stabilizing solvent like TFE.

Surprisingly, when the motif $(Lys)_3$–Ahx was conjugated to the C-terminus of the sequence 103–124, the obtained peptide **3** adopted a helical conformation both in methanol and in TFE, which was partially destabilized upon water addition (Fig. 2E and F). Thus, modifying the NES region at the C-terminus was structurally not equivalent to modifying it at the N-terminus, as only in the latter case the peptide was allowed to form a stable β-sheet structure. One reason might be the generation of unfavorable electrostatic interactions upon peptide-chain alignment, thus preventing the formation of β-sheets.

Based on the CD study of peptides **1**–**3**, we conclude that the isolated Id2 fragment containing the NES domain can form stable β-sheets or α-helices depending on the solvent. Of the two more soluble analogs, only the N-terminally modified one (**2**) presented conformational properties very similar to those of the unmodified peptide, whereas the C-terminally modified analog (**3**) lost the ability to form a β-sheet structure.

Beside the conformation of the Id2 NES region in solution, we also wanted to characterize its insoluble form. To do that, we analyzed the morphology of the aggregates formed by the NES peptide **1** and by the variant **2** by electron microscopy (variant **3** was not considered in this study, because it did not conserve the conformational features of the unmodified NES sequence). We prepared two samples of peptide **1** in methanol and methanol/water 80:20 (the high alcohol content was necessary to slow down the precipitation), while we dissolved the more hydrophilic peptide **2** in methanol/water 20:80. After incubation for 10 days or 1 month, the obtained suspensions were stained with a 2% aqueous solution of phosphotungstic acid (pH 7.2) and analyzed by EM. After 10 days, peptide **2** had formed fibrils with widths of 6–8 nm and lengths ranging from 60 to 200 nm (Fig. 5A). Fibrils characterized by a banding pattern with alternating light and dark zones of 8 and 3 nm heights, respectively, were also visible (Fig. 5B). Instead, isolated or assembled spherical particles (~10 nm in diameter) in conjunction with amorphous material were present in the sample of peptide **1** containing 80% methanol (Fig. 5C). Similar isolated spheres were detected for peptide **1** in methanol, although in this case the absence of water had strongly lowered the formation of deposits (Fig. 5D).

As it is known that β-sheet fibrils like those formed by amyloid peptides and proteins bind ThT [17], we investigated whether the Id2 NES aggregates also displayed this

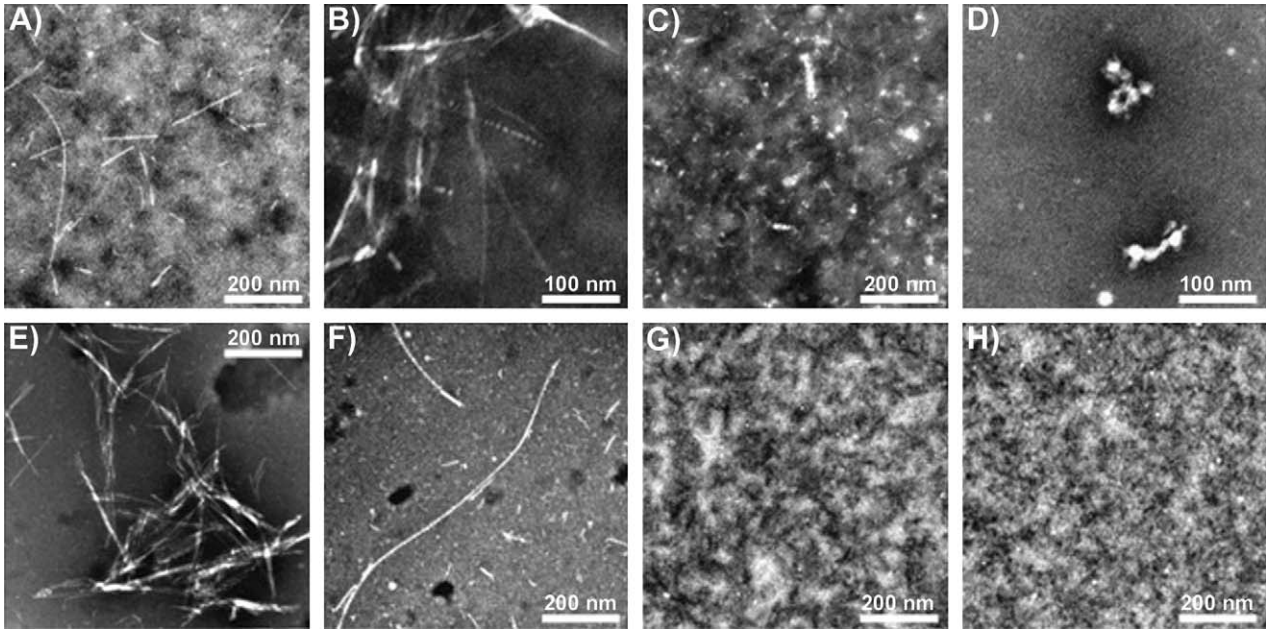

Fig. 5. EM images of the insoluble aggregates formed by peptides **1** and **2**. Negative staining was performed with a 2% aqueous solution of neutral phosphotungstic acid. Fibrils obtained by a 50 μM solution of peptide **2** in methanol/water 20:80 after 10 days (A,B) and 1 month (E,F). Spherical particles and amorphous material obtained by a 50 μM solution of peptide **1** in methanol/water 80:20 after 10 days (C) and 1 month (G). Spherical particles and amorphous material obtained by a 50 μM solution of peptide **1** in methanol after 10 days (D) and 1 month (H). See supplementary material for a magnified view of the EM pictures.

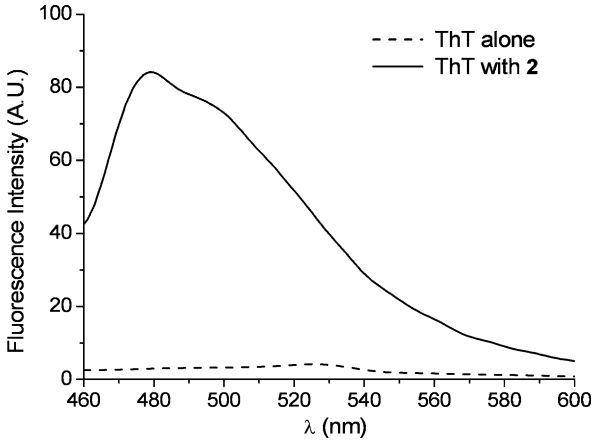

Fig. 6. Thioflavin T (ThT) assay. Fluorescence of ThT alone (5 μM in methanol/water 20:80) or mixed with a fibrillar sample of peptide **2** (50 μM in methanol/water 20:80). Twenty-five microliter of a 10-day-old peptide suspension was added to the ThT solution, which was then excited at 446 nm.

property. Indeed, by analyzing the 10-day-old samples of peptides **1** and **2**, we found that only peptide **2** increased the fluorescence emission of ThT at 484 nm after excitation at 446 nm (Fig. 6).

After 1 month, the peptide **2** fibrils reached up to 10 nm thickness and up to 1 μm length (Fig. 5E and F), while peptide **1** precipitated abundantly from methanol with and without 20% water, forming highly dense deposits which appeared to be mostly amorphous (Fig. 5G and H).

To control if the negative staining procedure could affect the morphology of the solid aggregates, the same 1-month-old samples above described were stained with a 2% aqueous solution of uranyl acetate (pH 4.1). In the case of peptide **2**, besides the already observed fibrils, well-defined spherical particles with diameters in the range of 12–30 nm were observed (Fig. 7A). Such granules were also characteristic of peptide **1** in 80% methanol (Fig. 7B), whereas the sample in methanol presented compact deposits of both fibrillar and amorphous material (Fig. 7C and D). These results suggest that the aggregates of the peptides **1** and **2** are pH-sensitive: in particular, the fibrils of peptide **2** seem to be unstable at low pH, where the formation of sphere-like oligomers is favored. This would be consistent with the previous antiparallel β-sheet model stabilized by electrostatic interactions between the positively charged lysine side chains and the negatively charged side chains of the C-terminal glutamic acids (Fig. 4). Such favorable interactions would be partly lost at pH values near 4, thus destabilizing the β-sheet aggregates.

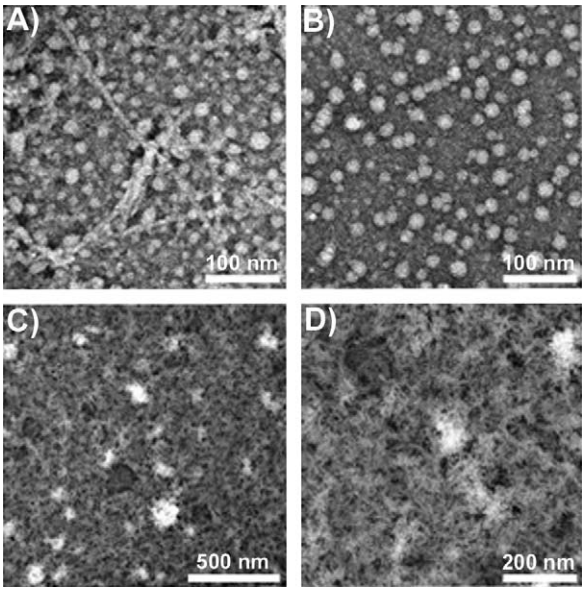

Fig. 7. EM images of the insoluble aggregates of peptides **1** and **2**. Negative staining was performed with a 2% aqueous solution of uranyl acetate after 1 month. Fibrils and spherical particles obtained by a 50 μM solution of peptide **2** in methanol/water 20:80 (A). Spherical particles obtained by a 50 μM solution of peptide **1** in methanol/water 80:20 (B). Clumped fibrils and amorphous material obtained by a 50 μM solution of peptide **1** in methanol (C,D). See the supplementary material for a magnified view of the EM pictures.

In the case of peptide **1**, the morphologies of the aggregates shown after acidic staining appeared more ordered than those shown after neutral staining: indeed, well-defined granular deposits and clumped fibrils were found for the sample in 20% water and in methanol, respectively. Thus, it is likely that the staining procedure performed at neutral pH partly converted the ordered aggregates to amorphous material, due to the increase in the peptide net charge after deprotonation of the aspartic and glutamic acid residues.

In conclusion, we have shown that the Id2 fragment 103–124 containing the leucine-rich NES motif adopts a β-sheet structure in methanol/water, from which it aggressively precipitates in amorphous, spherical or fibril-like forms depending on the environment. Thus far, there is no evidence that the Id2 protein is subjected to oligomerization/aggregation in vivo; however, it is known that Id2 can form homodimers stabilized by an intermolecular disulfide bond involving the HLH residue Cys42 and that the Ala/Cys42 mutation is deleterious for the Id2 activity [18]. Therefore, Id2 can undergo self-association, which might be a potential mechanism for auto-regulation or modulation of the inhibitory activity. Further studies are necessary to clarify whether the intrinsic propensity of the Id2 NES subdomain to aggregate has any significance for the folding and biology of this protein.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2006.05.108.

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