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Insights into the mechanism of Apoptin's exquisitely selective anti-tumor action from atomic level characterization of its conformation and dynamics

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ABSTRACT

Apoptin is a 121 residue protein which forms large, soluble aggregates and possesses an exceptionally selectively cytotoxic action on cancer cells. In the accompanying paper, we described the design, production and initial characterization of an Apoptin truncated variant called H_6-ApopΔProΔLeu. Whereas both the variant and wild type protein possess similar selective cytotoxicity against cancer cells following transfection, only the variant is cytotoxic when added externally. Remarkably, as observed by gel filtration chromatography and dynamic light scattering, H_6-ApopΔProΔLeu lacks the tendency of wild type Apoptin to form large aggregates, which greatly facilitated the study of its biological properties. Here, we characterize the conformation and dynamics of H_6-ApopΔProΔLeu. Using a battery of 2D, 3D and (4,2)D NMR spectra, the essentially complete ^{1}H, ^{13}C and ^{15}N resonance assignments of H_6-ApopΔ ProΔLeu were obtained. The analysis of these data shows that the variant is an intrinsically disordered protein, which lacks a preferred conformation. This conclusion is corroborated by a lack of protection against proteolytic cleavage and hydrogen/deuterium exchange. Moreover, the CD spectra are dominated by random coil contributions. Finally, ^{1}H-^{15}N NOE ratios are low, which indicates flexibility on the ps-ns time scale. Interestingly, H_6-ApopΔProΔLeu's intrinsically disordered ensemble is not significantly altered by the redox state of its Cys residues or by Thr phosphorylation, which has been proposed to play a key role in Apoptin's selective cytotoxicity. These results serve to better comprehend Apoptin's remarkably selective anticancer action and provide a framework for the future design of improved Apoptin variants.

Apoptin, a protein isolated from the chicken anemia virus, is cytotoxic against all tumor cell lines tested whereas it leaves normal cells unharmed [1–3]. In tumor cells, Apoptin appears to act by inducing apoptosis through a p53 independent pathway. Moreover, Apoptin does not cause any toxic effects, transforming activity or interferes with cell proliferation in normal human fibroblasts when expressed long term [4]. These remarkable properties have sparked increasing interest in this protein, as evidenced by the fact that the number of publications on Apoptin increases year after year. Different formulations of Apoptin are now being tested as cancer treatments and are advancing through clinical trials [5].

Composed of 121 amino acid residues, Apoptin is relatively small and contains a proline rich segment (PRS, residues 8–28) and a leucine rich segment (LRS, residues 33–46) which promote oligomerization [*see Ref.* [6] *and our accompanying paper, Ruiz-Martínez* et al., *submitted*] as well as motifs for nuclear localization (NLS, residues 82–88 & 111–121) and nuclear export (NES, 97–105) (see Fig. 1). Thr108, which is adjacent to the NES, can be phosphorylated by tumor specific kinases [7]. To date, two kinases have been reported to phosphorylate Apoptin, namely protein kinase Cß [8] and CDK2 [9], albeit depending on the type of tumor cell, different serine/threonine kinases must be implicated. As a result, the NES is masked

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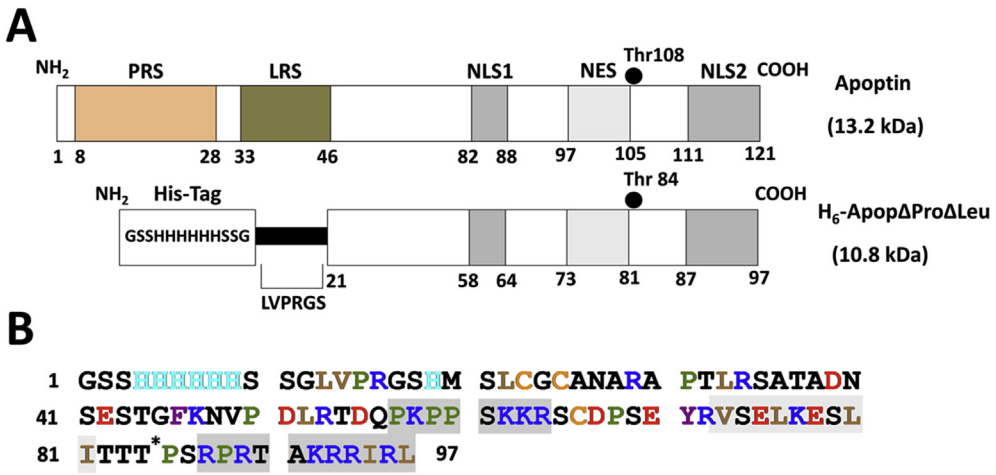

Fig. 1. Sequence of the H_6-ApopΔProΔLeu variant. **A**) Functional stretches in the sequence of Apoptin and description of the modifications introduced in H_6-ApopΔProΔLeu variant. Nuclear localization sequences (grey), the nuclear export sequence (pale grey), leucine rich segment (brown) and proline rich segment (orange) are indicated. The black circle indicates the position of phosphorylatable Thr108 in Apoptin. The sequence of the His-tag and the thrombin recognition site of H_6-ApopΔProΔLeu are indicated. **B**) H_6-ApopΔ ProΔLeu Apoptin variant's sequence. Anionic residues (Asp & Glu) are colored red and cationic residues (Arg & Lys) are colored blue. His residues, which bear a positive charge at pH 5.5 but become neutral above pH 6.5 are colored cyan. The sequence contains an excess of positively charged residues; in particular, the nuclear localization sequences are highly cationic: (Asp + Glu = 8; Arg + Lys = 17; Arg + Lys + His = 24). The construct's three Cys residues are colored orange and the ten aliphatic residues are colored brown. The sequence is rich in Pro (green), but is poor in aromatic residues; the lone Phe and Tyr residues are colored purple. Thr108 (Thr84 in this construct), whose specific phosphorylation by cancer specific kinases putatively masks the nuclear export sequence [7], is marked with an asterisk. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

which triggers a buildup of Apoptin in the tumor cell's nucleus. This buildup has been proposed to be the basis of Apoptin's selective antitumor cytotoxicity. This attractive hypothesis is supported by the observation that a Thr108 to Glu variant, which mimics the phosphorylated state of Thr, is cytotoxic to both cancer and normal cell lines [7]. The elimination of Thr108 does not completely abolish Apoptin's cytotoxicity since the preceding threonine (Thr107) is able to play this role, although the protein's activity is decreased [10,11]. Several studies have investigated the proteins that Apoptin interacts with and their roles in the apoptosis pathway triggered by Apoptin (see Ref. [12] for a recent review), and a low resolution structural model for full length Apoptin and its complex with the Bcr-Abl oncoprotein has been advanced [13]. However, up until now the conformation of Apoptin at atomic resolution has remained unknown, mainly because the protein tends to form large soluble aggregates that thwart characterization by high resolution techniques like X-ray crystallography or NMR. This limits our understanding of how the protein acts, how phosphorylation of Thr108 could affect its structure and the molecular details of its recognition and interaction with other proteins in the apoptosis pathway.

We have cloned and characterized a truncated variant of Apoptin called H_6-ApopΔProΔLeu that lacks the PRS and LRS and retains the wild type protein's selective cytotoxicity against tumor cells. This variant does not form large aggregates in solution (*see Ruiz-Martínez* et al., *submitted*) which makes its characterization using high resolution techniques possible. The main objective of this paper is to study the conformation and dynamics of this active Apoptin variant at atomic resolution using NMR spectroscopy and other methods. We also aim to determine whether the redox state of H_6-ApopΔ ProΔLeu's cysteine residues affects its structure and to test the effect of Thr phosphorylation on H_6-ApopΔProΔLeu's conformation.

1. Experimental procedures

1.1. Protein expression and purification

His-tagged Apoptin and its variants H_6-ApopΔPro and H_6-ApopΔProΔLeu were cloned and characterized as described in the submitted accompanying paper. For multiple dimensional, heteronuclear NMR spectroscopy, the variants' expression was induced in minimal media containing ^{13}C-glucose and ^{15}N ammonium chloride as the only source of carbon and nitrogen atoms, respectively, following previously published protocols [14]. The degree of ^{13}C and ^{15}N incorporation was judged by comparing the theoretical mass of the completely isotopically labeled protein to the mass determined experimentally using matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry at the Instituto de Química Física “Rocasolano”.

1.2. ANS binding

The fluorescence emission spectra of ANS (1-anilinonaphthalene-8-sulfonic acid) with and without protein were recorded over a range from 400 to 600 nm (slit width 10 nm) with an excitation wavelength of 350 nm (slit width 5 nm) on a LS-50B fluorimeter (Perkin-Elmer, USA). Protein samples were prepared at a final concentration of 1 mg/ml in 50 mM TrisHCl pH 8.0. ANS was filtered through 0.22 μm syringe filters, added to the protein solution at a final concentration of 50 μM and incubated for 20 min at 22 °C prior to analysis.

1.3. Resistance to proteinase K

All proteins were prepared to a final concentration of 10 μM in 50 mM TrisHCl, 150 mM NaCl pH 8.0. The digestion was carried out using Proteinase K (Roche, recombinant PCR grade) at a final concentration of 0.35 μM for 1 h at room temperature. PMSF (1.5 mM) was used to stop the digestion, and then the samples were analyzed by SDS-PAGE (15% polyacrylamide).

1.4. Conformational stability studies

With the objective of determining the conformational stability of the H_6-ApopΔProΔLeu variant, its UV absorbance at 280 nm using a Perkin-Elmer LS-50B spectrophotometer was monitored during heating (from 18 °C to 70 °C) as described in Ref. [15].