Apoptin's functional N- and C-termini independently bind DNA

S.R. Leliveld[a,1], R.T. Dame[a,2], J.L. Rohn[b], M.H.M. Noteborn[a,b], J.P. Abrahams[a,*]

[a] *Leiden Institute of Chemistry, Leiden University, PO Box 9502, 2300 RA Leiden, The Netherlands*
[b] *Leadd BV, Leiden, The Netherlands*

Received 30 October 2003; accepted 9 December 2003

First published online 18 December 2003

Edited by Vladimir Skulachev

Abstract Apoptin induces apoptosis specifically in tumour cells, where Apoptin is enriched in the DNA-dense heterochromatin and nucleoli. In vitro, Apoptin interacts with dsDNA, forming large nucleoprotein superstructures likely to be relevant for apoptosis induction. Its N- and C-terminal domains also have cell-killing activity, although they are less potent than the full-length protein. Here, we report that both Apoptin's N- and C-terminal halves separately bound DNA, indicating multiple independent binding sites. The reduced cell killing activity of both truncation mutants was mirrored in vitro by a reduced affinity compared to full-length Apoptin. However, none of the truncation mutants cooperatively bound DNA or formed superstructures, which suggests that cooperative DNA binding by Apoptin is required for the formation of nucleoprotein superstructures. As Apoptin's N- and C-terminal fragments not only share apoptotic activity, but also affinity for DNA, we propose that both properties are functionally linked.

Key words: Apoptin; Apoptosis; DNA interaction; Multimerisation; Protein-DNA superstructure

1. Introduction

Apoptin, a 121 amino acid protein derived from the chicken anaemia virus (CAV), has tumour-specific apoptosis-inducing activity [1,2]. In tumour cells, it predominantly co-localises with heterochromatin and nucleoli, followed by induction of apoptosis [3]. Extensive biophysical and in vivo studies with recombinant Apoptin protein fused to maltose-binding protein (MBP-Apoptin), which induces tumour-specific apoptosis like wild-type Apoptin [4], provide a feasible biophysical basis for its preference for these DNA-dense nuclear substructures. MBP-Apoptin consists exclusively of uniform, globular particles that are water soluble and contain 30–40 monomers [5,6], which cooperatively bind dsDNA with high affinity [3]. Moreover, up to 20 MBP-Apoptin multimers assemble with a continuous stretch of dsDNA into a higher-order superstructure in a highly cooperative manner [3,7]. Apoptin's multimerisation motif is located in its N-terminal 69 amino acids, and probably includes residues 32–46, which are predicted to form an amphipathic β-hairpin [7]. Apoptin's N-terminal domain is slightly acidic with a predicted p*I* of 6.5. The MBP fusion protein carrying Apoptin's highly positively charged C-terminal domain, residues 66–121 with a predicted p*I* of ~10, forms monomers and dimers/trimers without detectable traces of higher-order species [5].

Recently, Danen-van Oorschot et al. described that the presence of either Apoptin's N-terminal half or its C-terminal half is sufficient for apoptosis induction, albeit reduced compared to full-length Apoptin. In either case, a strong correlation between nuclear localisation and killing activity is observed [8].

In this study, we present our assessment of the biophysical and DNA-binding properties of the functional N-terminal and C-terminal halves of Apoptin. As it was shown earlier, an N-terminal MBP domain does not interfere with the biological activity of Apoptin [4], we used MBP-tagged mutants of Apoptin, containing residues 1–80, or residues 29–121, or residues 66–121. The MBP tag facilitates purification, stabilises the protein and enhances heterologous protein expression. Our data revealed that Apoptin fragments known to have a limited but pronounced apoptosis activity, associated with DNA, suggesting that Apoptin-DNA binding as such is an essential part of Apoptin's apoptosis activity, even though we did not observe the formation of pronounced superstructures.

2. Materials and methods

2.1. Cloning, expression and purification of Apoptin constructs

2.1.1. pMalTBVP3(1–80). The N-terminal (1–80) fragment of Apoptin (open reading frame nt 1–240) was amplified by polymerase chain reaction (PCR) from pET11aVP3 [7] and cloned into pET22b (Novagen) at *Nde*I/*Not*I, yielding pET22bVP3(1–80). Subsequently, the Apoptin(1–80) fragment, including a C-terminal hexahistidine tag and T7 terminator, was amplified by PCR from pET22bVP3(1–80) and cloned into the expression plasmid pMalTB *Bam*HI and *Sal*I [5], resulting in pMalTBVP3(1–80) encoding MBP fused to the Apoptin(1–80)-H_6 fragment.

2.1.2. pMalTBVP3(29–121). The (29–121) Apoptin fragment was amplified by PCR and cloned into pMalTB at *Bam*HI and *Sal*I, resulting in pMalTBVP3(29–121) encoding the MBP-Apoptin(29–121) fragment.

The expression plasmids pMalTBVP3(1–121), encoding the recombinant MBP fused to full-length Apoptin, and the expression plasmid pMalTBApoptin(66–121), encoding the MBP fusion of the C-terminal (66–121) Apoptin fragment, were described previously [5,7].

The expression and purification of the various recombinant pro-

*Corresponding author. Fax: (31)-71-5274357.
E-mail address: abrahams@chem.leidenuniv.nl (J.P. Abrahams).

[1] Present address: Institute of Neuropathology, Heinrich-Heine University, Düsseldorf, Germany.
[2] Present address: Physics of Complex Systems, Faculty of Sciences, Free University, Amsterdam, The Netherlands.

Abbreviations: CAV, chicken anaemia virus; DLS, dynamic light scattering; MBP, maltose-binding protein; SEC, size exclusion chromatography; SFM, scanning force microscopy

0014-5793/03/$30.00
doi:10.1016/S0014-5793(03)01465-0

teins, including DNA-affinity chromatography, has been performed as described by Leliveld et al. [3,5,7]. All purified proteins were stored in 20 mM HEPES pH 7.4, 10 mM NaCl, 1 mM EDTA at 4°C.

2.2. Dynamic light scattering (DLS) and scanning force microscopy (SFM)

All DLS measurements were performed on a DynaPro-MS/X (Protein Solutions Inc.). Experimental conditions were as described by Leliveld et al. [3,5,7]. Analysis of mutant Apoptin protein and DNA complexes thereof were analysed by SFM as described previously [3,7].

2.3. Size exclusion chromatography (SEC)

SEC was performed using a BioLogic LC system (Biorad). Columns: 1. Superose 6 HR 10/30 (Amersham), calibrated with a SEC calibration kit (Biorad) (670, 158, 44, 17, 1.35 kDa) and run in phosphate-buffered saline (PBS), 1 mM EDTA at room temperature, flow = 0.2–0.4 ml min^{-1}, sample load = 100 μl. 2. Superose 6 (prep grade) (1.6×26 cm) was packed in a XK16 column (Amersham), calibrated with SEC kit (Biorad) and run in PBS, 1 mM EDTA at room temperature.

2.4. Fluorescence measurements

The ethidium bromide exclusion assay was performed as described previously [3]. We determined the number of bound 12 bp fragments per multimer from the protein concentration needed to bind 50% of the fragments, which corresponded to 50% fluorescence quenching.

3. Results

3.1. Multimerisation of Apoptin truncation mutants

First, we examined the multimerisation capacity of the Apoptin truncation mutants. SEC and DLS analysis confirmed that both MBP-Apoptin(1–80)-H_6 and MBP-Apoptin(29–121) formed monodisperse multimers of about 500 ± 100 kDa and 450 ± 100 kDa respectively, implying a stoichiometry of about 8–10 monomers per multimer (data not shown). As also MBP-Apoptin(1–69) is known to multimerise, unlike MBP-Apoptin(66–121) [5], we propose that Apoptin's multimerisation motif resides within residues 29–66, which include a predicted amphipathic β-hairpin spanning residues 32–46. However, since multimers of MBP-Apoptin(1–80)-H_6 and MBP-Apoptin(29–121) both contained fewer monomers than full-length MBP-Apoptin, other parts of the protein must also be involved in multimerisation.

3.2. DNA-binding characteristics of MBP-Apoptin truncation mutants

Next, we evaluated DNA-binding kinetics of the three Apoptin mutants and compared them with those of wild-type, multimeric Apoptin, which cooperatively binds dsDNA. For this purpose, we used an assay for quantifying dsDNA binding based on the exclusion of ethidium bromide from dsDNA, as described previously [3]. All three mutants expelled ethidium bromide from dsDNA in solution (Fig. 1A,B), albeit less efficiently than MBP-Apoptin(1–121). A titration of a 12 bp dsDNA fragment yielded K_D values of 60 ± 5 and 25 ± 5 nM for the multimeric mutants containing residues 1–80 and 29–121, respectively, whereas non-multimerising MBP-Apoptin(66–121) had a K_D of 400 ± 50 nM. When we titrated a dsDNA fragment of 177 bp, none of the mutants showed the cooperativity of binding characteristic

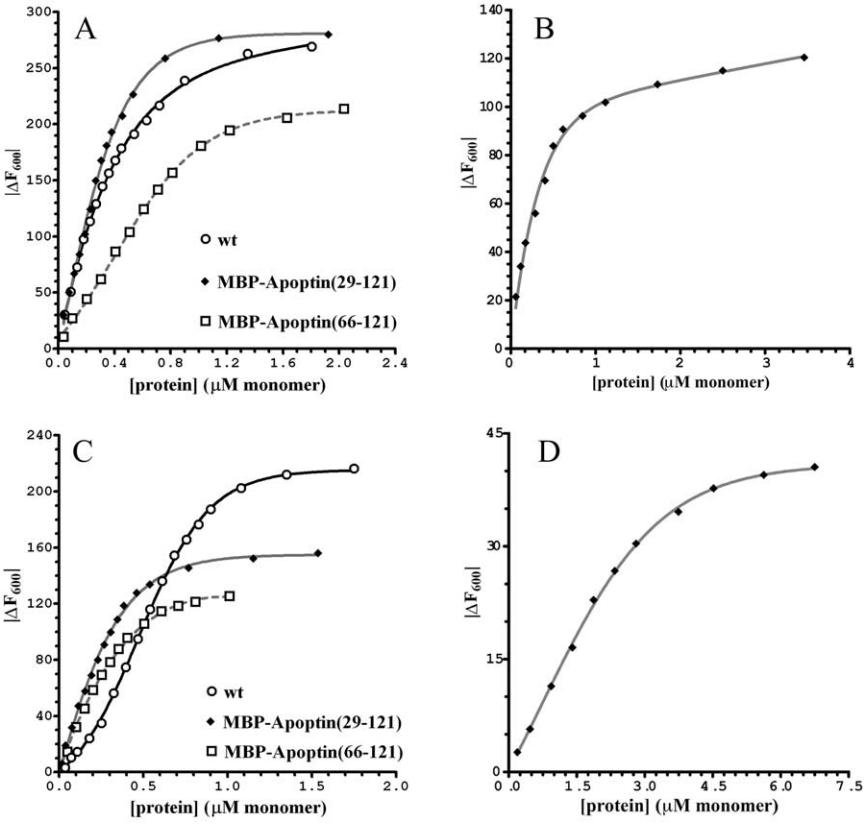

Fig. 1. DNA-binding characteristics of Apoptin mutants. A: Titration of a 12 bp blunt-ended dsDNA fragment (1 μg ml^{-1}, 130 nM fragment) with MBP-Apoptin(1–121) (wt), MBP-Apoptin(66–121) and MBP-Apoptin(29–121); $|\Delta F_{600}|$ = reduction in EB fluorescence at 600 nm. B: Idem, for MBP-Apoptin(1–80)-H_6. C: Titration of a 177 bp blunt-ended dsDNA fragment (1 μg ml^{-1}, 8.7 nM fragment) with wild-type MBP-Apoptin (wt), MBP-Apoptin(66–121) and MBP-Apoptin(29–121). D: Idem, for MBP-Apoptin(1–80)-H_6.

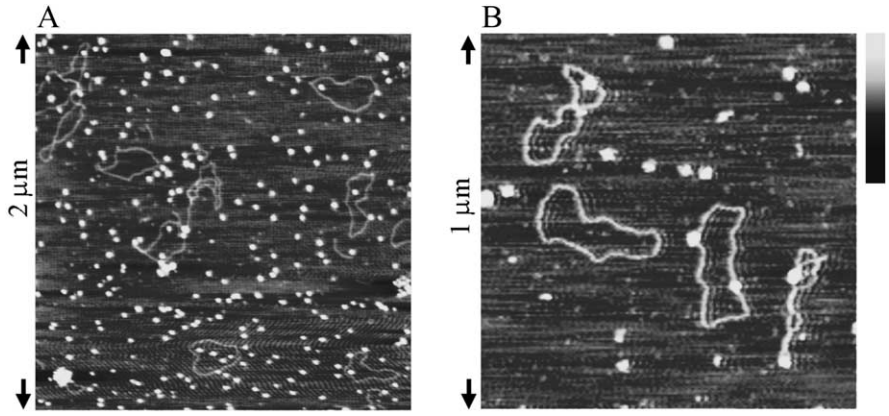

Fig. 2. Morphology of mutant Apoptin particles alone and bound to DNA, determined by SFM. The DNA template used here was DNaseI-nicked pUC19 (2.7 kb). A: MBP-Apoptin(29–121), combined with pUC19 at a DNA to protein ratio of 370 bp per multimer, 2×2 µm surface area. B: MBP-Apoptin(1–80)-H_6, combined with pUC19 at a DNA to protein ratio of 15 kb per multimer, 1×1 µm surface area. The height is indicated in grey scale, the bar is from 0.0 (black) to 1.5 nm (white).

of full-length MBP-Apoptin (Fig. 1C,D). The results show that all three Apoptin mutants bound DNA in solution, but, unlike wild-type Apoptin, not in a cooperative fashion.

3.3. *Nucleoprotein superstructure formation of MBP-Apoptin truncation mutants with DNA*

Finally, we examined whether the Apoptin truncation mutants, like full-length Apoptin, formed higher-order superstructures upon association with naked dsDNA. According to DLS analysis, MBP-Apoptin(29–121) formed higher order complexes with DNA, that were comparable in size to those formed by full-length MBP-Apoptin. For instance, the superstructure of MBP-Apoptin(29–121) multimers with a linear 2.6 kb dsDNA fragment reached a hydrodynamic diameter of 88 ± 4 nm, compared to 86 ± 4 nm for full-length MBP-Apoptin. However, MBP-Apoptin(29–121)·DNA superstructures formed less efficiently than full-length MBP-Apoptin·DNA superstructures. In addition to measuring the formation of these superstructures by DLS, we visualised them by SFM (Fig. 2A), where the lower efficiency of superstructure formation was evidenced by the high abundance of MBP-Apoptin(29–121) multimers that had not bound to DNA. In comparison, full-length non-complexed MBP-Apoptin(1–121) multimers are far less abundant under comparable conditions [3]. DLS indicated that the MBP-Apoptin(1–80)-H_6 mutant did not form superstructures, which was confirmed by SFM, showing only singly bound MBP-Apoptin(1–80)-H_6·DNA complexes (Fig. 2B). Likewise, we failed to detect MBP-Apoptin(66–121)·DNA superstructures by SFM analysis (data not shown).

4. Discussion

Here, we showed that Apoptin's N- and C-terminal domains can independently bind naked dsDNA, in spite of their very different primary sequences. Together with in vivo observations reported earlier [8], this suggests that nucleic acid binding plays an important role in the cell-killing mechanism of Apoptin. In human tumour cells synthesising truncated Apoptin(1–69), the mutant protein localises mainly in the cytoplasm, without inducing apoptosis [8]. In contrast, when Apoptin(1–69) is fused to a heterologous nuclear localisation signal the resulting translocation of the mutant Apoptin to the nucleus is followed by a markedly increased induction of apoptosis in tumour cells only. These observations underline that Apoptin or its fragments induce apoptosis only when present in the nucleus – where it can interact with the cellular DNA.

The multimers formed by MBP-Apoptin(1–69)-H_6 are of the same size as those formed by MBP-Apoptin(1–121) [5]. As also MBP-Apoptin(29–121) multimerised in experiments reported here, the multimerisation centre can be narrowed down to residues (29–69), comprising a putative amphipathic β-hairpin including residues 32–46. However, as both MBP-Apoptin(29–121) and MBP-Apoptin(1–80)-H_6 truncation mutants aggregated into smaller complexes than full-length MBP-Apoptin, optimal multimerisation is determined by residues flanking the hairpin motif. As MBP-Apoptin(66–121) did not multimerise but was shown to be active in apoptosis induction and DNA binding, we conclude that even a monomeric interaction with DNA may be sufficient for Apoptin's activity.

Although both N-terminal MBP-Apoptin(1–80)-H_6 and C-terminal MBP-Apoptin(66–121) bound at least two dsDNA docecamers per multimer, their DNA-binding sites did not cooperate efficiently when binding to a 177 bp fragment. The non-cooperativity in DNA binding of all examined truncation mutants is in marked contrast to the cooperative binding of full-length MBP-Apoptin multimers [3]. Our results suggest that the full-length Apoptin protein multimer has been optimised towards efficient, high-affinity DNA binding [3], and that both its N-terminal half as well as its basic C-terminal half are required for cooperativity. Furthermore, we conclude that the distinct formation of Apoptin-DNA superstructures require structural motifs from both N- and C-terminal domain, since neither N- nor C-terminal truncation allowed efficient formation of these superstructures. Our data indicate that cooperative DNA binding by Apoptin is required for the formation of nucleoprotein superstructures.

Although our data suggest a strong correlation between direct DNA interaction and induction of apoptosis, it is likely that tumour-specific cellular factors are essential in orchestrating cell death. The requirement of such factors could explain why Apoptin fused to a heterologous nuclear location signal and entering the nucleus of normal cells does not induce apoptosis [8].

In conclusion, our in vitro studies indicated that both Apoptin's N-terminal and C-terminal halves independently bind DNA, albeit with lower affinities than full-length Apoptin. As both N- and C-terminal halves can also independently induce apoptosis (with a reduced activity compared to full-length Apoptin), we propose that Apoptin's capacity to bind DNA is closely linked to its biological activity, possibly through interfering with chromatin structure and/or dynamics.

References

[1] Noteborn, M.H.M., Danen-van Oorschot, A.A.A.M. and van der Eb, A.J. (1998) Semin. Virol. 8, 497–504.
[2] Noteborn, M.H.M. (2002) Cancer Drug Discovery and Development, pp. 275–287, Humana Press Inc., Totowa, NJ.
[3] Leliveld, S.R., Dame, R.T., Mommaas, M.A., Koerten, H.K., Wyman, C., Danen-van Oorschot, A.A.A.M., Rohn, J.L., Noteborn, M.H.M. and Abrahams, J.P. (2003) Nucleic Acid Res. 31, 4805–4813.
[4] Zhang, Y.-H., Leliveld, S.R., Kooistra, K., Molenaar, C., Rohn, J.L., Tanke, H.J., Abrahams, J.P. and Noteborn, H.H.M. (2003) Exp. Cell Res. 289, 36–46.
[5] Leliveld, S.R., Zhang, Y.-H., Rohn, J.L., Noteborn, M.H.M. and Abrahams, J.P. (2003) J. Biol. Chem. 278, 9042–9051.
[6] Leliveld, S.R., Noteborn, M.H.M. and Abrahams, J.P. (2003) Eur. J. Biochem. 270, 3619–3627.
[7] Leliveld, S.R. (2003) Ph.D. Thesis, Leiden University, Leiden, The Netherlands.
[8] Danen-van Oorschot, A.A.A.M., Zhang, Y.-H., Leliveld, S.R., Rohn, J.L., Seelen, M.C.M.J., Bolk, M.W., van Zon, A., Erkeland, S.J., Abrahams, J.P., Mumberg, D. and Noteborn, M.H.M. (2003) J. Biol. Chem. 278, 27729–27736.