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FEBS Letters 342 (1994) 185–188

FEBS 13861

FEBS LETTERS

Biochemical properties of attachment region binding protein ARBP

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Received 27 January 1994

Abstract

ARBP (attachment region binding protein) is an abundant nuclear protein that specifically binds to matrix/scaffold attachment regions (MARs/SARs). Here we show by gel filtration and gradient sedimentation that ARBP has an elongated shape. The sedimentation coefficient was determined as only 2.1 S. Furthermore, limited proteolysis of ARBP in situ (in isolated nuclei) with several proteases generated limiting resistant peptides from 14.5 to 18 kDa, that retained the ability to bind MARs specifically. This indicates that these peptides encompass the DNA binding domain of ARBP.

Key words: Chromatin organization; ARBP; DNA binding domain; Sedimentation coefficient; Stokes radius

1. Introduction

Interphase chromatin is partitioned into topologically sequestered loop-domains [1]. There is evidence that these domains correspond to units of packaging as well as to units of gene regulation. It is thought that chromatin loops are generated by attachment of specific sequences (MARs/SARS) to the nuclear matrix/chromosomal scaffold [2]. MARs/SARs have been characterized as AT-rich sequences, at least several hundred base pairs in length, that localize in some well studied cases to domain boundaries [3–5] (for reviews see [6,7]). In an attempt to analyse the attachment of MARs to nuclear matrix, we used a Southwestern blotting assay to identify proteins that specifically bind to MARs [8]. ARBP (attachment region binding protein) is one of the identified proteins. It is phylogenetically conserved, from Drosophila to human, and recognizes several sites within the 5′-MAR of the chicken lysozyme gene. Other laboratories have reported on the selective binding of additional known as well as newly discovered proteins to MARs: DNA topoisomerase II, lamin B_1, histone H1, SAT-B1, and SAF-A [9–13].

In many DNA binding proteins, the ability for specific DNA binding resides in a protein domain, that can fold and act independently of the remaining molecule. In a search, whether ARBP also contains such a domain, we performed limited proteolysis of ARBP in situ, i.e. in purified nuclei. We demonstrate that various proteases generated 14.5–18 kDa peptides that retained the ability to specifically recognize MARs. Furthermore, the behaviour of ARBP in gel filtration and glycerol gradients implies that the protein has an elongated shape.

2. Materials and methods

2.1. Materials

Preparation of nuclear extracts from hen oviducts and purification of ARBP was performed as described previously [8].

2.2. Gel filtration and glycerol gradient centrifugation

Gel filtration was performed by chromatography on a Superose 6 HR 10/30 column (Pharmacia) at 20°C in 0.05% Triton X-100, 20 mM Tris-HCl, pH 7.5, 300 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 10% glycerol. Nuclear extract was applied to the column together with molecular weight standard proteins (Pharmacia): porcine thyroglobulin (669 kDa, 85 Å), horse spleen ferritin (440 kDa, 61 Å), bovine liver catalase (232 kDa, 52.5 Å), and bovine heart lactate dehydrogenase (140 kDa, 36 Å) (values given by the manufacturer). The marker contents were analysed on SDS-polyacrylamide gels that were stained with Coomassie brilliant blue. The elution profile of ARBP was determined by a Southwestern blotting assay using the MAR fragment H1-HaeII as a probe [8].

To determine the sedimentation coefficient, purified ARBP was layered together with marker proteins (Boehringer: horse cytochrome *c*, bovine chymotrypsinogen A, ovalbumin, bovine serum albumin) on top of a linear 10–30% glycerol gradient in 20 mM Tris-HCl, pH 7.5, 230 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol [14]. The gradient was centrifuged in a Beckman SW60 rotor at 56,000 rpm for 20 h at 4°C. Fractions (200 μl) were collected from the bottom of the tube, and the sedimentation profile of ARBP and marker proteins was determined by Southwestern blotting assay and polyacrylamide gel electrophoresis, respectively.

Two-dimensional polyacrylamide gel electrophoresis was performed using the O'Farrell minigel system [15,16] with pI markers from Bio-Rad (hen egg white conalbumin, 6.0–6.6; bovine muscle actin, 5.5; bovine carbonic anhydrase, 5.9–6.0).

2.3. Limited proteolysis

Nuclei were purified from laying hen oviducts following the procedure described by Phi-Van and Strätling [5]. Nuclear suspensions

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SSDI 0014-5793(94)00246-R

(1.5 mg DNA/ml) in 20 mM Tris-HCl, pH 7.5, 50 mM NaCl, 3 mM $MgCl_2$ were incubated for 10 min at 25°C in 100 μl aliquots with either one of the following endoproteinases: bovine pancreatic elastase (Sigma), elastase from human leucocytes (Sigma), L-1-tosylamide-2-phenylethyl chloromethyl ketone-treated bovine pancreatic trypsin (Sigma), endoproteinase lys-C (Boehringer), and endoproteinase arg-C (Boehringer). Following digestion, reactions were stopped by the addition of 20 μl of buffer E (1.6 M NaCl, 20 mM Tris-HCl, pH 7.5, 3 mM $MgCl_2$, 0.5% 2-mercaptoethanol, 7.5 mM phenylmethylsulfonyl fluoride, 0.1 mg/ml Aprotinin (Sigma)). Samples were then extracted by vigorous shaking at 4°C for 45 min. After clarification in an Eppendorf centrifuge, 40 μl of the supernatants were mixed with 10 μl of 5-fold sample buffer (62.5 mM Tris-HCl, pH 7.5, 2% sodium dodecyl sulfate, 10% glycerol, 140 mM 2-mercaptoethanol, 0.12% Bromophenol blue). Identification of ARBP and MAR binding fragments therefrom utilized a Southwestern blotting assay [8] with electrophoretic separation on 8.5% (Fig. 3, panel G) or 13% (remaining panels) polyacrylamide gels.

3. Results and discussion

3.1. ARBP has an elongated shape

Gel filtration and glycerol gradient sedimentation were used to examine the biochemical properties of ARBP. On Superose-6 chromatography in 300 mM NaCl with standard globular proteins, ARBP eluted as a symmetrical profile with an elution volume, which corresponds to that of a globular protein with a mass of about 420 kDa (Fig. 1). However, in glycerol gradients with internal marker proteins purified ARBP sedimented rather slowly, at a position between those of cytochrome *c* (12.3 kDa) and chymotrypsinogen A (25 kDa). From four independent experiments, a sedimentation coefficient of only 2.13 S (S.D. ± 0.086) was determined (Fig. 2). Thus the sedimentation rate was much lower, but on the other hand the apparent mass on gel filtration was much higher than expected for a globular ≈90 kDa protein. Our results therefore indicate that ARBP has an elongated shape. There are several precedents for elongated nucleic acid-binding proteins, for example the mammalian poly(A)-binding protein II [18] and the yeast RNA polymerase II initiation factor a [19]. By comparison with the marker proteins, we calculated for ARBP an apparent Stokes radius of about 6.0 nm. For several rod-shaped proteins, however, a discrepancy between the apparent Stokes radius determined by gel filtration and the Stokes radius derived by sedimentation measurements has been reported [20–22]. Thus, the value of 6.0 nm determined for ARBP is likely to be an underestimate.

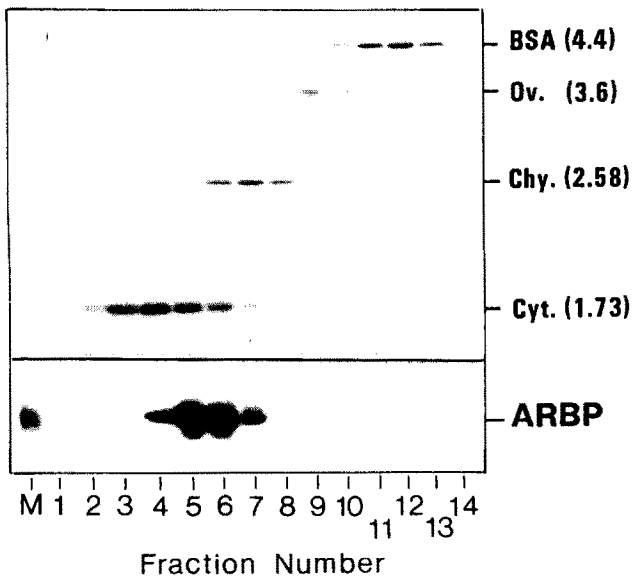

Fig. 1. Gel filtration of ARBP. Nuclear extract was chromatographed on a Superose 6 column and eluted fractions were analysed for the content of ARBP using a Southwestern blotting assay. The elution positions of protein standards are indicated: thyroglobulin (Ty), ferritin (Fe), catalase (Ca), and lactate dehydrogenase (LDH).

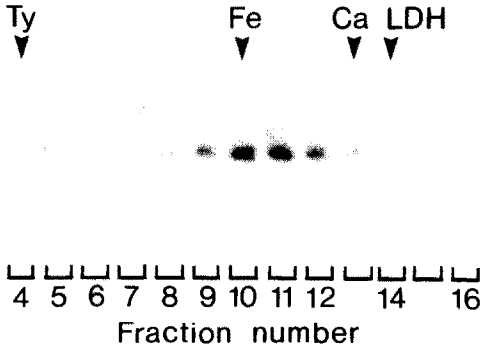

Fig. 2. Sedimentation coefficient of ARBP. Purified ARBP was sedimented in a glycerol gradient together with protein molecular weight standards (BSA, bovine serum albumin; Ov., ovalbumin; Chy., chymotrypsinogen A; Cyt., cytochrome *c* with the S values [17] shown in brackets). Fractions were collected and analysed for the contents of marker proteins by SDS-PAGE (upper panel) and for that of ARBP by Southwestern blotting assay (lower panel). Lane M contains purified ARBP.

Furthermore, ARBP appears to be structurally very stable: not only is it stable to heat denaturation at 90°C, but it can be isolated with its native DNA-binding properties from trichloroacetic acid precipitates (data not shown). The isoelectric point of ARBP was determined by two-dimensional gel electrophoresis as approximately 6.1 (data not shown).

3.2. Domain organization of ARBP

The domain organization of ARBP was analysed by limited proteolysis of oviduct nuclei with either one of several endoproteases followed by extraction with 320 mM NaCl. ARBP and proteolytic fragments therefrom, that retain the ability to specifically interact with MARs, were then identified by Southwestern blotting assay using the MAR probe H1-HaeII [8]. Upon incubation of isolated nuclei with increasing concentrations of pancreatic elastase, ARBP initially cleaved into two fragments with MAR binding activity, a fragment with an apparent size of approximately 81 kDa and a peptide with apparently 17 kDa (Fig. 3, panels A, B and C). At high concentrations of elastase the 17 kDa peptide was processed into a 15 kDa species. The results from two experiments

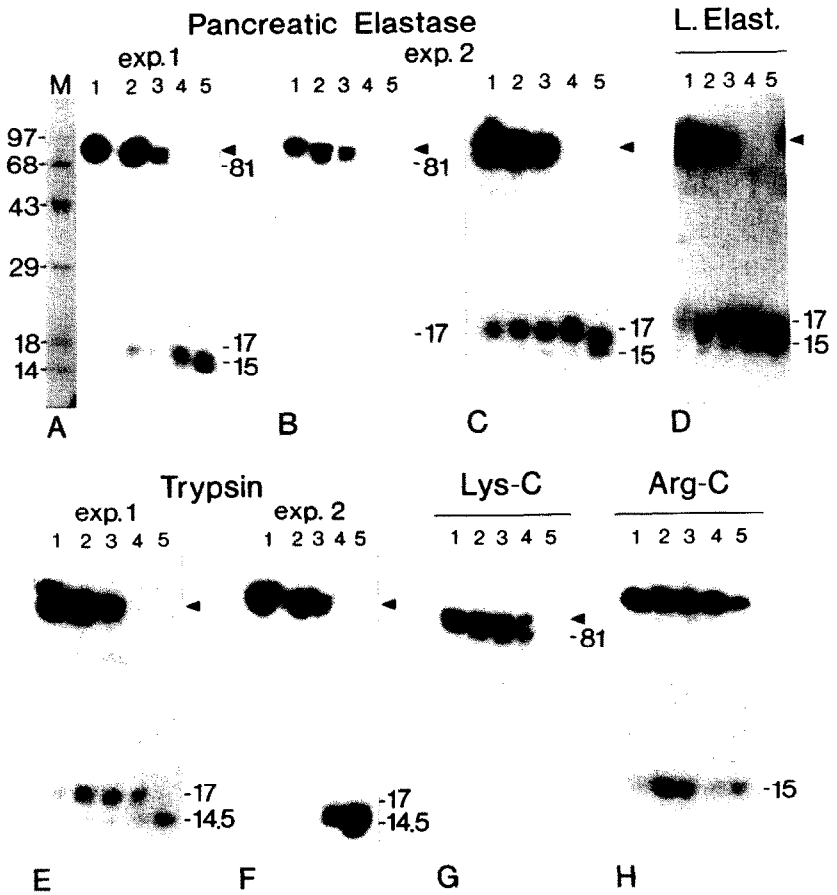

Fig. 3. Limited proteolysis of ARBP in situ. Suspensions of oviduct nuclei were treated with 0 (lanes 1), 1x (lanes 2), 5x (lanes 3), 10x (lanes 4) or 50x (lanes 5) units of pancreatic elastase (panels A, B and C), trypsin (panels E and F) or lys-C (panel G), or with 0 (lanes 1), 1x (lanes 2), 2x (lanes 3), 10x (lanes 4) or 20x (lanes 5) units of elastase from human leucocytes (L. Elast.) (panel D) or arg-C (panel H), where x equals 8.9×10^{-4} for pancreatic elastase, 6×10^{-3} for elastase from leucocytes, 1.3×10^{-4} for trypsin, 2×10^{-5} for lys-C, and 1×10^{-3} for arg-C. Reactions were terminated, and ARBP and proteolytic fragments therefrom retaining MAR binding activity were extracted and identified by electrophoretic separation and incubation of the blotted proteins with the MAR probe H1-HaeII. For digestion with pancreatic elastase and trypsin two experiments are shown (exp. 1 and exp. 2). Panel C is a stronger exposure relative to panel B. M, marker proteins (molecular masses in kDa). The arrowheads mark the bands corresponding to uncleaved ARBP, and proteolytic fragments retaining MAR binding activity are indicated with their approximate molecular mass (in kDa).

are shown: the 81 kDa intermediate is best resolved in panel A, lane 3, (exp.1) and panel B, lanes 2 and 3, (exp. 2), while the 17 kDa and 15 kDa peptides are clearly separated in panel C, lanes 3–5, (exp. 2). Occasionally we observed small amounts of the 17 kDa peptide already in the non-digested sample (see panel C, lane 1), probably arising from slight endogenous proteolysis. Peptides with 17 kDa and 15 kDa retaining MAR binding activity were also generated by digestion with elastase from human leucocytes (panel D), demonstrating that these peptides are not products from contaminating trypsin activity in the pancreatic elastase preparation. Digestion with trypsin also produced two MAR binding peptides with apparent sizes of approximately 17 kDa and 14.5 kDa (panels E and F). Again the results from two experiments are shown: in the first one, the two resistant peptides are clearly resolved (panel E, lanes 2–5). Incubation with the lysine-specific endoproteinase lys-C gave rise to a fragment with the apparent size of ≈81 kDa (panel G), while the arginine-specific endoproteinase arg-C generated a peptide with ≈15 kDa in size (panel H). Proteinase K, finally, produced an approximately 18 kDa peptide with MAR binding activity (data not shown).

These results show that digestion of ARBP with endoproteinases with different enzymatic specificities generated limiting resistant peptides with apparent sizes from 14.5 to 18 kDa, that retained the ability to recognize the MAR fragment H1-HaeII of the chicken lysozyme gene. Most likely these peptides are derived from the same region of ARBP and encompass the DNA binding domain of the protein. Interestingly, we observed that ARBP was completely cleaved into 15–17 kDa peptides by trypsin, when there was yet no sign of proteolysis of the bulk of the nuclear proteins as examined on stained gels (data not shown). This suggests that ARBP is located at proteolytically exposed sites in chromatin. This agrees with the relative ease with which the protein can be extracted from nuclei (100–300 mM NaCl) [8].

Acknowledgements: We thank M. Harbers for assistance in the gel filtation experiments. This work was supported by grants to W.H.S. from the Deutsche Forschungsgemeinschaft.

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