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Purification and characterization of p27, a protein from hepatocyte chromatin

Evidence suggesting that it binds selectively to guanine-rich single-stranded DNA

Michal A. Rahat, Michael Fry*

Unit of Biochemistry, The Bruce Rappaport Faculty of Medicine, Technion - Israel Institute of Technology, POB 9649, Bat Galim, Haifa 31096, Israel

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A protein designated p27 that binds preferentially to single-stranded DNA rich in guanine tracts was purified to near homogeneity from rabbit hepatocyte non-histone protein extract. Purified p27 migrated as a 27 kDa polypeptide on denaturing SDS-PAGE and displayed a native molecular mass of ~ 155 kDa on Sephadex G-150 or Sepharose 6B-Cl gel filtration columns. Gel shift analysis indicated that maximum binding of p27 to single-stranded DNA required the presence of tracts of four or more contiguous guanine residues. The lowest found dissociation constant, 1.4×10^{-8} M/l, was for single-stranded DNA that contained a $(dG)_{17}$ run.

DNA binding protein; Guanine-rich DNA; Single-stranded DNA

1. INTRODUCTION

Guanine-rich clusters appear in DNA at multiple locations in the genome such as telomers [1–3], regulatory regions of various genes [4–6], variable number of tandem repeats (VNTR) minisatellites [7,8], and the immunoglobulin switch region [9]. Some of these stretches possess the potential to form unusual structures, such as triple helix at $(dG)_n \cdot (dC)_n$ tracts [10,11], or tetrahelical DNA at regions of short runs of contiguous guanine residues [12,13].

Some proteins have been described that specifically bind to single- or double-stranded guanine-rich stretches or to the unusual structures that they form. A 100 kDa protein from *Oxytricha nova* [14] and an analogous protein from *Euplotes crassus* [15], bind to the single-stranded telomeric repeat 5′-d[T_2G_4]-3′ and protect it in vitro from nucleolytic degradation. A mouse hepatic nuclear protein, sTBP, similarly binds to a single-stranded telomeric sequence [16]. The chicken erythrocyte protein, BGP1, binds with a high affinity to single-stranded, guanine-rich tracts within the regulatory region of the β^A globin gene [17]. Recently we purified a protein, termed *QUAD*, from rabbit liver chromatin, that binds specifically to the quadruplex form of the IgG switch region sequence, 5′-d[TACAGGGGAGC-TGGGGTAGA]-3′, but not to its single-stranded form [13].

Here we report the isolation of a new protein from rabbit hepatocyte, designated p27, that binds preferentially to single-stranded, guanine-rich DNA but not to its double- or tetrahelical forms.

*Correspondng author. Fax: (972) (4) 510 735.

Abbreviations: DE-52, (diethylaminoethyl)cellulose-52; DTT, dithiothreitol; HEPES, (*N*[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid]); P-11, phosphocellulose 11; MalNet, *N*-ethylmaleimide; PMSF, phenylmethylsulfonylfluoride.

2. MATERIALS AND METHODS

2.1. *Materials*

Radioactively labeled 5′-[γ-^{32}P]ATP (~ 3,000 Ci/mmol) was the product of Amersham, UK. HPLC-purified synthetic oligonucleotides were synthesized by Operon Technologies, Almada, CA. Enzymes and DTT, MalNet, PMSF, leupeptin, glycine and cytochrome *c* were obtained from Sigma, St. Louis, MO, except for bacteriophage T4 polynucleotide kinase and the Klenow fragment of DNA polymerase I which were provided by United States Biochem., Cleveland, OH. DE-52, P-11 and DE-81 filter paper were the products of Whatman, UK. Dynabeads M-280-Strepavidin were supplied by Dynal, Skoyen, Norway.

2.2. *Gel mobility shift assay*

Protein fractions were incubated at 42°C for 20 min with 1 ng of a 5′-^{32}P-labeled oligonucleotide as specified in 10 mM HEPES, pH 7.5, 2 mM DTT, 50 mM NaCl, 0.1 mM EDTA, 17% glycerol. DNA–protein complexes were subsequently separated for 90 min at 12.5 V/cm on 5% polyacrylamide gels (acryl:bis 30:1.2) in Tris-glycine buffer (6 mM Tris-HCl, 47 mM glycine, 0.2 mM EDTA, pH 8.3). After electrophoresis the gel was dried on DE-81 filter paper, autoradiographed and amounts of free and protein-bound DNA were calculated from Cerenkov radioactivity in cut bands and the predetermined specific activity of the labeled DNA. 1 U of p27 was defined as the amount of protein that bound 100 pg of 5′-d[AATTC$(G)_{17}$]-3′ under the conditions specified above.

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2.3. *Purification of p27*

Salt extract of non-histone nuclear proteins was prepared from isolated nuclei of female, New Zealand white rabbit hepatocytes as described [20]. Preparation of the extract and all the subsequent steps of chromatography were conducted at 4°C. Briefly, livers were excised from the sacrificed, 2–2.5 kg body weight rabbits, washed in 0.3 M sucrose, 4 mM $CaCl_2$, dried lightly and resuspended in 9 vols. of the same solution. Cells were disrupted by 40 strokes with a B-type pestle of a Dounce homogenizer and the extract was filtered through four layers of cheesecloth. Nuclei were collected by centrifugation at $1,000 \times g$ for 10 min and the nuclear pellet was resuspended in an equal volume of 25 mM Tris-HCl buffer, pH 9.0, 0.8 M NaCl, 0.5 mM DTT, 1 mM EDTA, 5 μg/ml leupeptin, 1 mM PMSF and 40% glycerol. After 15 min stirring in the cold, the suspension was Dounce homogenized, centrifuged at $12,000 \times g$ for 20 min, the pellet was resuspended and similarly extracted twice more. Residual DNA was removed from the pooled extracts by chromatography through a 0.4 M NaCl-equilibrated DEAE column. The extract was dialyzed against DE buffer (25 mM Tris-HCl pH 8.2, 0.5 mM DTT, 1 mM EDTA, 20% glycerol) and loaded onto a DE buffer-equilibrated DE-52 column at a ratio of 5 mg protein/1 mg of packed resin. Proteins were batchwise eluted by 2 column vol. washes each of 50, 100, 150, 200, 250, 300 and 400 mM KCl in DE buffer. Aliquots from each eluted fraction were dialyzed against DE buffer and assayed by mobility shift for the binding of 5′-[^{32}P]d(G-C)$_{10}$-3′. To stabilize the labile binding activity, cytochrome *c* was added at 0.2 mg/ml to the protein fractions from this purification step onward. Fractions that contained binding activity were dialyzed overnight against P buffer (25 mM potassium phosphate (KP_i), pH 8.0, 0.5 mM DTT, 1 mM EDTA, 20% glycerol) and loaded onto a P buffer-equilibrated P-11 column at a ratio of 2 mg protein/ml of packed resin. Proteins were eluted by a 10-column vol. gradient of 25–400 mM KP_i in P buffer. Collected fractions were dialyzed against DE buffer and assayed for 5′-[^{32}P]d(G-C)$_{10}$-3′ binding activity. Aliquots, 0.5 ml each, of pooled p27 activity were mixed with biotinylated 5′-d[AATTC(G-C)$_{10}$G]-3′ bound to 0.6×10^8 magnetic Dynbeads M280. Biotinylation of the oligomer was conducted by Klenow polymerase-catalyzed incorporation of Bio-11-dUTP. Attachment of the DNA to the strepavidin-coated beads was carried out according to the manufacturer's instructions. Proteins were batchwise eluted by washing the beads with 0.1 ml aliquots of DE buffer that contained 0.01–2.0 M NaCl. Following dialysis against DE buffer, eluted fractions were assayed by gel mobility shift for 5′-[^{32}P]d[AATTC(G)$_{17}$]-3′ binding activity.

2.4. *Determination of the molecular mass of p27*

Molecular mass of denatured, DNA–Dynabeads-purified p27 was determined by its relative electrophoretic migration in 10% SDS-PAGE. In parallel, the identity of this protein was verified by gel mobility shift analysis. Molecular mass marker proteins were those previously listed [13]. Molecular mass of non-denatured Dynabeads-purified p27 was determined by its gel partition coefficients (K_{av}) of the mobility shift-identified DNA binding activity on Sepharose 6B-CL (0.7×46.5 cm) or Sephadex G-150 (1.0×50.5 cm) columns. Molecular mass marker proteins were those previously listed [13].

2.5. *Determination of the amount of protein*

The Bio-Rad (Richmond CA) protein assay kit was used to determine the amount of protein.

3. RESULTS

3.1. *Purification of p27*

Two non-histone proteins that bound 5′-d(G-C)$_{10}$-3′ were detected in the 150 mM KCl eluate of a DE-52 column by gel-shift analysis (data not shown). These two proteins were resolved from each other by subsequent P-11 column chromatography. Whereas one protein was eluted from P-11 by 100–180 mM KP_i, an activity later identified as p27 was eluted by 180–245 mM KP_i (results not shown). The P-11 fraction of p27 was further purified by affinity binding to biotinylated 5′-d[AATTC(G-C)$_{10}$G]-3′ attached to Dynal M-280 magnetic beads. Due to the low yield of p27 after this purification step, repeated cycles of binding and elution had to be performed to obtain amounts of protein detectable by Coomassie blue staining. Activity of p27 was eluted from the beads by 0.1–2.0 M NaCl in DE buffer (Fig. 1, upper panel). Experiments indicated that the addition of increasing amounts of p27 to a constant

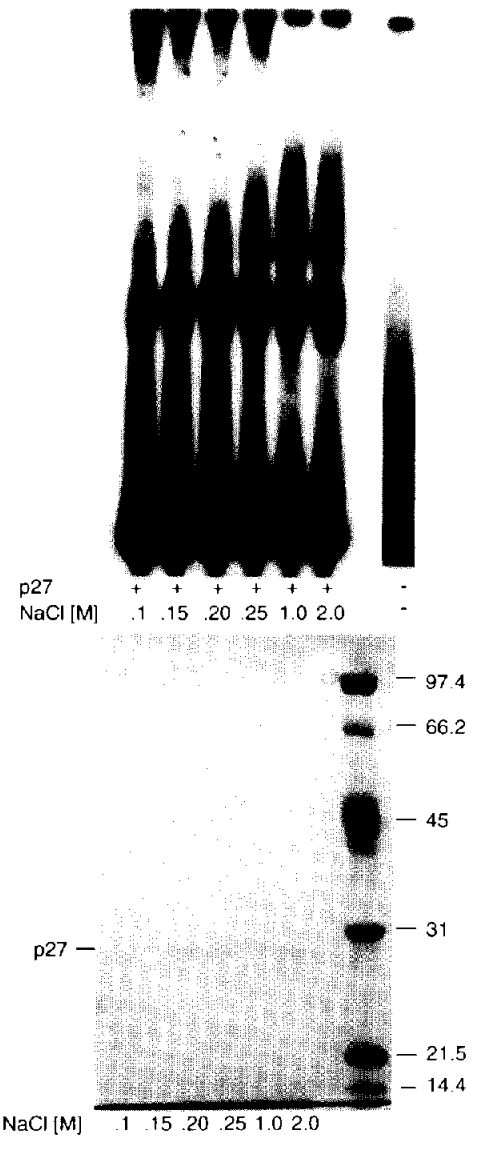

Fig. 1. Purification of p27 by DNA–Dynabead affinity binding. The P-11 fraction of p27 was purified by binding to, and salt elution from, Dynabead-immobilized 5′-d[AATTC(G-C)$_{10}$G]-3′. Fractions were pooled from six consecutive rounds of binding and elution. (Upper panel) Mobility shift analysis of the binding of salt eluted fractions to 5′-^{32}P-labeled d[AATTC(G)$_{17}$]-3′. (Lower panel) 10% SDS-PAGE and Coomassie blue staining of proteins eluted from DNA–Dynabead.

amount of 5′-[^{32}P]d[AATTC(G)$_{17}$] and separation of protein–DNA complexes by mobility shift electrophoresis, resulted in the appearance of more heavily retarded bands that presumably represented multi-molecular p27 DNA aggregates (data not shown). Hence, results presented in the upper panel of Fig. 1 suggest that the activity of eluted p27 was highest in the 0.25 2.0 M NaCl fractions, as indicated by the elevated amounts of p27–DNA aggregates. As shown in the lower panel of Fig. 1, a single major band of 27 kDa was seen in fractions extracted with 0.1–2.0 M NaCl, and its amount was highest in the 0.25–2.0 M NaCl fractions. Additional more heavily stained bands that were present in every fraction originated from a contamination in the loading buffer. The correlation between binding activity and presence of the 27 kDa protein band suggested that the 27 kDa polypeptide represented the binding protein.

3.2. *Chemical-physical properties of p27*

Table I summarizes some properties of purified p27. The presented results indicate that p27 was heat- and trypsin labile but that it did not require a nucleic acid component for its activity. The partial sensitivity of p27 to 10 mM MalNet may indicate that reduced sulfhydryl groups are only indirectly involved in the interaction of the protein with DNA. The finding that the only polypeptide present in affinity-purified p27 preparations has a molecular mass of 27 kDa (Fig. 1), and the observed molecular mass of ~ 155 kDa for the non-denatured protein (Table I), suggested that the native protein was probably a homohexamer.

Table I

Chemical-physical properties of p27

Treatment/property	Residual activity/value
None	100.0
Trypsin[a]	0.0
MalNet[c]	49.2
Micrococcal nuclease[b]	126.0
85°C, 10 min[d]	11.0
Denatured molecular mass[e]	27.0
Native molecular mass	130; 170[f]
	150; 150[g]

Activity of p27 was quantified by mobility shift analysis of complex formation with 5′-[^{32}P]d[AATTC(G)$_{17}$]-3′.

[a] Trypsin, 0.3 mg/ml, was incubated with 8.5 U of p27 in a volume of 15 μl at 37°C for 60 min and the reaction was terminated by adding 1.5 mg/ml of soy bean trypsin inhibitor.

[b] Micrococcal nuclease, 41 μg/μl, was incubated with 6.4 U of p27 in 15 μl at 37°C for 30 min in the presence of 1 mM $CaCl_2$. Digestion was terminated by adding EDTA and thymidine 3′,5′ diphosphate to a final concentration of 2.6 mM each.

[c] MalNet, 10 mM, was added to 8.4 U of p27 at 4°C for 15 min and the reaction was terminated by adding 66 mM DTT.

[d] Relative to 100% binding activity observed after incubation of p27 at 42°C for 10 min.

[e] Average of two determinations by SDS-PAGE.

[f] Results of two independent determinations by Sepharose 6B gel filtration.

[g] Results of two independent determinations by Sephadex G-150 gel filtration.

Table II

DNA sequence specificity of p27 binding

Competitor DNA	Residual binding (%)
5′-d[AATTC(G)$_{17}$]-3′	0
5′-d[AATTC(A)$_{16}$G]-3′	104
5′d[AATTC(T)$_{16}$G-3′	108
5′-d[AATTC(C)$_{16}$G]-3′	93
5′-d[AATTC(G-A)$_{12}$G]-3′	123
5′d[AATTC(C-T)$_{12}$G]-3′	108
5′-d[GTAAAACGACGGCCAGT]-3′	109
5′-d[AATTC(G-C)$_{10}$G]-3′	61
5′-[TACAGGGGAGCTGGGGTAGA]- 3′	0
5′-d[$(T_2G_4)_4$]-3′	2

DNA Dynabead-purified p27 was incubated under standard binding conditions in a mixture that contained 5′-[^{32}P]d[AATTC(G)$_{17}$]-3′ and a 30-fold molar excess of unlabeled competing oligomer. Complexing of p27 with the labeled oligomer was quantified after its resolution by gel-shift electrophoresis. The notations (G-A)$_{12}$, (C-T)$_{12}$, (G-C)$_{10}$ and $(T_2G_4)_4$ designate strings with total lengths of 24, 24, 20 and 24 nucleotides, respectively.

3.3. *DNA binding by p27*

Activity of p27 was initially detected by its capacity to bind d(G-C)$_{10}$. However, binding competition experiments showed that the protein bound more tightly to other guanine-rich sequences, with 5′-d[AATTC(G)$_{17}$]-3′ being the preferred DNA ligand. As shown in Table II, when added at a 30-fold molar excess, unlabeled oligomers that lack guanine runs did not compete with 5′-[^{32}P]d[AATTC(G)$_{17}$]-3′ on binding to p27. By contrast, oligomers that contained clusters of guanine residues, 5′-d[TACAGGGGAGCTGGGGTAGA]-3′ or 5′-d[$(T_2G_4)_4$]-3′ efficiently chased 5′[^{32}P]d[AATTC(G)$_{17}$]-3′ from its complex with p27. Interestingly, whereas the alternating purine oligomer 5′-d[AATTC(G-A)$_{12}$G]-3′ did not compete with 5′-d[AATTC(G)$_{17}$]-3′ on its binding to p27, partial displacement was obtained with the alternating purine–pyrimidine oligomer, 5′-d[AATTC-(G-C)$_{10}$G]-3′. To quantify the affinity of p27 for the different guanine-rich oligomers, their dissociation constants, K_d, were determined. As shown in Table III, p27 displayed its highest affinity towards 5′-d[AATTC-(G)$_{17}$]-3′ which contained the longest-tested guanine tract. Dissociation constants of oligomers that contained four and two (dG)$_4$ runs were, respectively, 6 and 31-fold higher than that of 5′-d[AATTC(G)$_{17}$]-3′. The lowest-measured affinity was for 5′-d[AATTC-(G-C)$_{10}$G]-3′, which had a dissociation constant 236-fold higher than that of 5′-d[AATTC(G)$_{17}$]-3′ (Table III). It appeared, therefore, that p27 bound preferentially to single strands that contained runs of contiguous guanine residues, and that its affinity to such sequences was directly related to the length of the guanine run. To

Table III

Dissociation constants of p27 binding to guanine-rich DNA oligomers

Oligomer	K_d (M/l)	Relative affinity
5′-d[AATTC$(G)_{17}$]-3′[a]	$1.4 \times 10^{-8} \pm 0.1 \times 10^{-8}$	1.0
5′-d[$(T_2G_4)_4$]-3′[b]	$1.8 \times 10^{-7} \pm 0.9 \times 10^{-7}$	0.16
5′-d[TACAGGGGAG-CTGGGGTAGA]- 3′[c]	4.3×10^{-7}	0.032
5′-d[AATTC$(G\text{-}C)_{10}$]-3′[b]	$3.3 \times 10^{-6} \pm 0.5 \times 10^{-6}$	0.0042

Increasing amounts of each oligomer and an added constant amount of tracer 5′-^{32}P-labeled oligomer were incubated with 0.7 U of p27 under standard binding conditions. DNA–p27 complexes were resolved by mobility shift electrophoresis and amounts of free and bound DNA were determined (see section 2 and [13]). The dissociation constant is the reciprocal of the negative value of the slope of resulting Scatchard plots.

[a] Average of three determinations.

[b] Average of two determinations.

[c] A single determination.

determine whether p27 is also capable of binding double-stranded DNA, 5′-d[AATTC$(G)_{17}$]-3′ and 5′-d[AATTC$(C)_{17}$G]-3′ were annealed, internally labeled as described previously [18], and incubated with increasing amounts of p27. Gel-retardation analysis did not reveal significant formation of complexes between this DNA and p27 (data not shown). Furthermore, double-stranded poly(dG)·poly(dC) did not compete with 5′-[^{32}P]d[AATTC$(G)_{17}$G] on binding to p27 (data not shown). To examine whether p27 binds guanine-rich tetrahelical DNA, mobility shift analysis was used to examine the binding of p27 to the quadruplex parallel form of 5′-d(TACAGGGGAGCTGGGGTAGA)-3′ [13]. We found that p27 failed to bind quadruplex DNA under conditions that enabled its efficient binding by rabbit liver *QUAD* protein [13] (results not shown). It thus appeared that p27 bound to guanine-rich single-stranded DNA but not to double- or tetra-stranded DNA.

4. DISCUSSION

Evidence presented in this work suggests that the hepatic protein, p27, is a guanine-rich DNA binding protein. However, formal proof that p27 indeed binds to such DNA must await DNA–protein molecular crosslinking studies. The several previously characterized proteins that were found to associate with guanine-rich single-stranded DNA differ from the presently described hepatic p27. First, the ~ 155 and 27 kDa native and denatured molecular sizes, respectively, of p27 differed from those of the 43 and 55 kDa subunit heterodimer telomeric DNA binding protein from *Oxytricha nova* [14] and from the monomeric 50 kDa telomere binding protein from *Euplotes crassus* [15]. Likewise, p27 was structurally distinct from the hepatic 37 kDa telomeric polypeptide, sTBP [16], and from the 66 kDa chicken erythrocyte poly(dG) binding protein, BGP1 [6,17]. Second, the DNA binding specificity of p27 differed from those of most other guanine-rich DNA binding proteins. Whereas p27 bound to runs of contiguous guanine residues in oligomers with different nucleotide sequences (Tables II and III), *Oxytricha nova* [14], *Euplotes crassus* [15] and mouse [16] telomeric DNA binding proteins are sequence-specific. Most similar to p27 is the chicken erythrocyte protein, BGP1, that binds with a high affinity ($K_d = 1.3 \times 10^{-9}$ M/l) to a $(dG)_{16}$ cluster within the regulatory region of the gene coding for β^A globin [6,17]. Thus, although p27 differed structurally from the 66 kDa chicken BGP1 and had a ~ 10-fold lower affinity for $(dG)_{17}$, these two proteins share a similar sequence preference. Last, in contrast to some proteins, such as Myo D [19] and *QUAD* [13], that bind tetrahelical forms of guanine-rich DNA, p27 failed to associate with quadruplex DNA.

In the past we described two sequence-specific single-stranded DNA binding proteins that stimulated in vitro copying of the bound sequences by alleviating pausing of DNA polymerases and increasing their processivity [18,20–22]. To learn whether p27 might be a member of this family of polymerase-stimulating proteins, we applied primer extension analysis to examine the capacity of this protein to enhance Klenow polymerase or calf thymus polymerase α-catalyzed copying of the bacteriophage M13 DNA template that contained different $(dN)_{16}$ inserts, N being A, C, G, T, (C-T) or (A-G) [23]. In contrast to the previously described factor D [18,20,21] and factor C [22], p27 failed to remove replicative barriers within the inserts and did not enhance DNA synthesis with any of the tested templates (results not shown). Thus, p27 is not a DNA polymerase-stimulating protein and its function(s) remain to be uncovered.

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