

Basic homopolyamino acids, histones and protamines are potent antagonists of angiogenin binding to ribonuclease inhibitor

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Abstract A radio-ribonuclease inhibitor assay based on the interaction of ¹²⁵I-angiogenin with ribonuclease inhibitor (RI) was used to detect pancreatic-type ribonucleases and potential modulators of their action. We show that highly basic proteins including the homopolypeptides poly-arginine, poly-lysine and poly-ornithine, core histones, spermatid-specific S1 protein and the protamines HP3 and Z3 were strong inhibitors of angiogenin binding to RI. A minimum size of poly-arginine and poly-lysine was required for efficient inhibition. The inhibition likely resulted from direct association of the basic proteins with the acidic inhibitor, as RI bound to poly-lysine and protamines while ¹²⁵I-angiogenin did not. Antagonists of the angiogenin-RI interaction are potential regulators of either angiogenin-triggered angiogenesis and/or intracellular RI function, depending on their preferential target.

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Key words: Angiogenin; Ribonuclease inhibitor; Poly-arginine; Poly-lysine; Histone; Protamine

1. Introduction

The RNase superfamily consists of low molecular weight proteins structurally related to RNase A [1,2]. Several of these RNases are inhibited in vitro by a unique 51 kDa polypeptide known as the RNase inhibitor (RI). This is the case for pancreatic RNase [3,4], angiogenin [3], eosinophil-derived neurotoxin (EDN; also referred as to liver or placental RNase) [5,6], eosinophil cationic protein (ECP) [7], tumor RNase [8], bovine seminal ribonuclease (BS-RNase) monomers [9] and mammalian orthologous RNases [10,11]. The inhibitor binds tightly at a ratio of one to one with RNases [10,11]. Its crystal analysis has shown a horseshoe shape. In this conformation, the inhibitor binds to an extensive surface of RNase A and angiogenin that includes part of their substrate binding sites [12,13].

On the basis of the angiogenin interaction with RI, an in vitro binding assay has been developed to detect members of the RNase superfamily whatever their catalytic activities [14]. Using this assay, we now report that basic homopolyamino acids as well as the lysine- and/or arginine-rich proteins histones and protamines, although structurally unrelated to RNases, are potent competitors of angiogenin binding to

RI. Like poly(Lys) and protamines, these competitors probably exert their effect by direct binding to the inhibitor. These results indicate that basic RNA or DNA binding proteins represent potential physiological inhibitors of RI and therefore local regulators of RNase functions.

2. Materials and methods

2.1. Materials

Human recombinant angiogenin was from Rhône-Poulenc Rorer ([Met⁻¹] angiogenin) or R. Shapiro ([< Glu¹] angiogenin). Bovine RNase A was from Calbiochem (San Diego, CA, USA). Protein concentration was determined by spectrophotometric absorption at 280 nm for angiogenin ($\epsilon = 12\,500\text{ M}^{-1}\text{ cm}^{-1}$) and at 278 nm for RNase A ($\epsilon = 9800\text{ M}^{-1}\text{ cm}^{-1}$) [5,15]. Restrictocin was from J. Davies. EDN and ECP were a gift from G. Gleich and BS-RNase was from G. D'Alessio. Native human RI was either supplied from Pharmacia (Uppsala, Sweden) or purified from erythrocytes [16], the two protein inhibitors giving essentially similar results. The activity of RI is reported in units (U), one unit being the amount required to inhibit 50% of 5 ng of RNase A in an assay using wheat germ RNA [16]. Protamine Z3 and spermatid-specific protein S1, both from dogfish, and alkylated human protamine HP3 were obtained according to reported procedures [17–19]. Calf thymus crude histone fractions were prepared according to the methods described by Johns [20,21]. Histones H2A, H2B, H3 and H4 were further purified by gel filtration on Biogel P60 (H2A, H2B) or Biogel P10 (H3, H4). Protamine and histone concentrations were determined by the Bio-Rad protein assay using BSA as standard. Poly(Lys) (4–15 kDa)-agarose, protamine-agarose, *para*-hydroxymercuribenzoate (*p*-HMB), bovine serum albumin (BSA), recombinant human lysozyme, arginine, lysine, putrescine, spermidine, spermine and homopoly-L-amino acids were from Sigma Chemical Co. (St Louis, MO, USA). Protein A-agarose and hydrogen peroxide (H₂O₂) were from Pierce (Rockford, IL, USA).

2.2. Radio-ribonuclease inhibitor assay (RRIA)

Angiogenin was iodinated using the chloramine-T method [22]. ¹²⁵I-Angiogenin was then loaded on a column containing 0.2 ml of heparin-Sepharose (Pharmacia). After a wash with MOPS-buffered saline (MBS, 20 mM MOPS, 130 mM NaCl, 3 mM KCl, pH 7.2) containing 1 mg/ml BSA, the iodinated protein was eluted in MBS, 1 M NaCl, 1 mg/ml BSA, and desalted in MBS, 1 mg/ml BSA on a PD10 column (Pharmacia). The specific radioactivity of the labelled protein was between 220 000 and 450 000 cpm/ng (1.5–3 Ci/μmol). RRIA was performed at 20°C essentially as described [14]. ¹²⁵I-Angiogenin (7000–15 000 cpm) and the test samples were diluted in RRIA buffer (MBS, 1 mg/ml BSA, 5 mM EDTA, 0.02% NaN₃; final volume 460 μl). Then, 0.015 U RI in 20 μl MBS, 5 mM DTT, pH 7.2 was added and incubation was continued for 10 min. ¹²⁵I-Angiogenin•RI complexes were precipitated in 50% ammonium sulfate in the presence of the globulin fraction of fetal calf serum [14]. Free ¹²⁵I-angiogenin was measured by counting aliquots of the supernatants. The data were analyzed using the RIA program operated by a 1275 MiniGamma counter (LKB). Experiments were performed at least twice in duplicate or triplicate.

2.3. Binding of RI to poly(Lys) and protamines

The interaction of RI with poly(Lys) and protamines was studied by incubating 0.12 U of RI in the presence of a given amount of

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Abbreviations: BS-RNase, bovine seminal ribonuclease; ECP, eosinophil cationic protein; EDN, eosinophil-derived neurotoxin; RI, ribonuclease inhibitor; RNase A, bovine ribonuclease A; poly(Lys), poly-lysine; poly(Arg), poly-arginine; poly(Orn), poly-ornithine

poly(Lys) or protamines coupled to beaded agarose. The reaction was run in polypropylene tubes for 15 min at 20°C in 200 μ l of RRIA buffer, 5 mM DTT. The tubes were centrifuged and 50 μ l aliquots of supernatant were incubated with 10000 cpm 125 I-angiogenin for 10 min. RI was then quantified using the RRIA procedure described above. RI bound to protein-agarose was released after two washes in RRIA buffer, 5 mM DTT and incubation for 15 min in the same buffer containing 2 M NaCl. 125 I-Angiogenin did not significantly bind to poly(Lys)-agarose or protamine-agarose in RRIA buffer. Control experiments were carried out using protein A coupled to agarose.

3. Results

3.1. Radio-ribonuclease inhibitor assay

125 I-Angiogenin binding to RI was quantified using differential precipitation of bound vs. free 125 I-angiogenin at 50% ammonium sulfate saturation. In the absence of RI, 90–95% of the radioactivity was recovered in the soluble fraction. In the presence of RI, 125 I-angiogenin precipitation increased with the RI/angiogenin ratio. Approximately 50% precipitation of the tracer was induced by 0.015 U RI, maximum activity being obtained with ~ 0.03 U RI (Fig. 1A). Thus, the RI/angiogenin ratio used in this assay was nearly identical to the RI/RNase A ratio required for enzymatic inhibition in ribonucleolytic assays [16]. The thiol reagent *p*-HMB abolished this effect, in keeping with its ability to inactivate RI in ribonucleolytic assays [23] and to dissociate RI•angiogenin complexes [24]. Likewise, H_2O_2 interfered with the formation of 125 I-angiogenin•RI complexes (Fig. 1B). However, the presence of DTT (0.2 mM) and BSA (which probably acted here as a radical scavenger) strongly limited the effect of H_2O_2 as a large amount of the oxidant was required for inhibition. Other thiol compounds (L-cysteine and glutathione) also protected RI from H_2O_2 -mediated oxidation (not shown).

3.2. RRIA detects RNases of the pancreatic RNase superfamily

RRIA was performed using the RI concentration required to bind 50–70% of the angiogenin tracer. The specificity of the assay was assessed by adding angiogenin-related RNases as potential competitors. As expected, 125 I-angiogenin•RI complex formation was gradually inhibited in the presence of in-

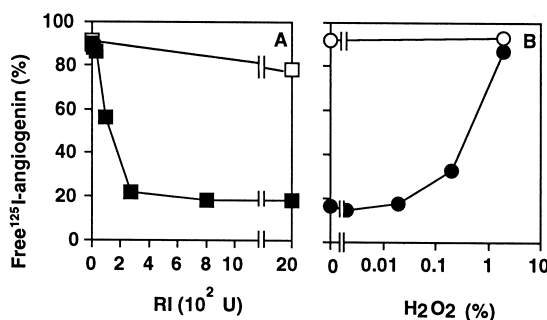


Fig. 1. Effect of *p*-HMB and H_2O_2 on the binding of 125 I-angiogenin to RI. 125 I-Angiogenin (25 μ g, 10000 cpm) was incubated with RI for 10 min at 20°C in the standard assay mixture. Separation of bound and free ligand was carried out in 50% saturated ammonium sulfate, and free 125 I-angiogenin was quantified by determining the percentage of total radioactivity present in the supernatant. A: The assay was performed in the presence of increasing amounts of RI, in the presence (□) or absence (■) of 1 mM *p*-HMB. B: A similar experiment was performed in the presence (●) or absence (○) of 0.03 U RI with increasing amounts of H_2O_2 (a 1% hydrogen peroxide solution corresponds to 376 mM H_2O_2). Each result is the mean of duplicate determinations that differed by less than 5%.

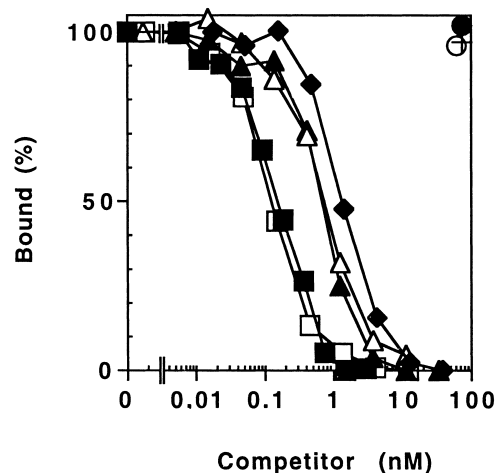


Fig. 2. Competitive binding RRIA. Competition for 125 I-angiogenin binding to 0.015 U RI by (■) [Met⁻¹] angiogenin, (□) RNase A, (Δ) EDN, (▲) ECP, (◆) BS-RNase, (●) alkylated angiogenin; (○) restrictocin and (+) human lysozyme. The results are expressed as the percentage of a control in which half the 125 I-angiogenin bound to RI. Values are the means of duplicate determinations that differed by less than 5%. The experiment was repeated three times with similar results. The expression of the protein concentrations is based on the following molecular masses (in Da): angiogenin, 14100; RNase A, 13700; EDN, 15460; ECP, 15570; BS-RNase, 27200; lysozyme, 14000; restrictocin, 16840.

creasing amounts of unlabeled angiogenin. 10 pmol of [Met⁻¹] angiogenin (Fig. 2) or [$<\text{Glu}^1$] angiogenin (not shown) was detected, the inhibitory concentration (IC_{50}) of both molecules being 140 pM. Other RNases related to angiogenin competed strongly for the binding of 125 I-angiogenin to RI. The most potent antagonist (apart from angiogenin itself) was RNase A (IC_{50} of 120 pM), in keeping with the ability of RI to inhibit the catalytic activity of the enzyme [3,4]. IC_{50} values for angiogenin and RNase A did not differ significantly. The neurotoxin EDN, whose ribonucleolytic [5,6] and biological [25] activities are inhibited by RI, prevented 125 I-angiogenin binding with an IC_{50} of 0.7 nM. The behavior of the closely related protein ECP was similar. Finally, BS-RNase was also a potent competitor ($\text{IC}_{50} \sim 1.3$ nM). Conversely, angiogenin inactivated by reduction and alkylation, the basic protein lysozyme and the unrelated RNase restrictocin failed to compete at concentrations of 60–75 nM. The specific binding of basic RNases ($\text{pI} > 9$) to RI ($\text{pI} \sim 4.7$) was thus not solely determined by ionic interactions and required an ordered three-dimensional conformation of the RNases [12,13].

The IC_{50} values thus depend on the RNase and probably reflect differences in association rates (k_2/K_1) rather than association constants (K_a), as the assays were not performed in conditions of equilibrium (owing to the instability of the inhibitor relative to the extremely low dissociation rate of RNase RI•complexes) [3,4]. Other factors such as polymerization of RNase subunits may contribute to IC_{50} variations, as illustrated here by the moderate effect of BS-RNase. In fact, competition with BS-RNase was unexpected as the native dimeric form of the enzyme is not inhibited by RI. This observation probably resulted from the existence of BS-RNase monomers generated by the presence of DTT in the reaction mixture [9].

3.3. Basic homopolyamino acids, histones and protamines inhibited 125 I-angiogenin binding to RI

Several lysine/arginine residues of RNase A contribute to its binding to RI. Although most of these basic residues are in unconserved positions in other RI-interacting RNases, they play a key role in the binding of angiogenin (and perhaps EDN) to the inhibitor [12,13,26]. This suggests that other basic polypeptides could interfere with RI and act as competitors for RNase binding. Indeed, poly(Arg), poly(Lys) and poly(Orn) inhibited 125 I-angiogenin binding to RI, with IC_{50} values in the 4–300 nM range (Fig. 3A). In contrast, no competition was observed with poly(Asn), which suggests that the cationic charge was a determining factor for effective inhibition. Because the polyamines putrescine, spermidine and spermine did not interfere to a measurable extent in the assay (not shown), the influence of cationic homopolymers was also assessed as a function of their size. As shown in Fig. 3B, the size of both poly(Arg) and poly(Lys) correlated with their ability to compete, the most potent effects being observed with the largest polymers. The 92 kDa poly(Arg) ($IC_{50} \sim 1$ M) was almost as potent as ECP and EDN ($IC_{50} \sim 0.7$ nM) and only seven-fold less active than angiogenin and RNase A (~ 0.14 nM). IC_{50} values were also in the nM range with lysine polymers of high molecular mass, although poly(Lys) was less active than poly(Arg). In comparison, arginine, lysine and the lysine pentamer did not compete significantly, and the lysine heptamer gave an IC_{50} value of 100 μ M.

We then considered the possibility that natural lysine- and/or arginine-rich proteins have similar properties by testing histones, protamines and the spermatid-specific S1 protein. As shown in Table 1, the results were consistent with those observed with poly(Arg) and poly(Lys): the higher the basic amino acid content, the stronger the competitive effect. The strong activity of H3, compared with H4, might be related to its higher arginine content. Finally, the protein Z3 was a less potent inhibitor than the protamine HP3; although Z3 is even closer to the poly(Arg) structure (65% arginine residues vs. 52%), its shorter size could be a limiting factor for inhibition (see Fig. 3B).

3.4. Association of RI with poly(Lys) and protamines

The activity of basic homopolymers, histones and protamines probably resulted from their direct interaction with RI, as the inhibitor bound to either poly(Lys) or protamines coupled to agarose beads (Fig. 4) while 125 I-angiogenin did not (not shown). The binding was somewhat specific as protein A-agarose did not retain RI. Binding was also reversible

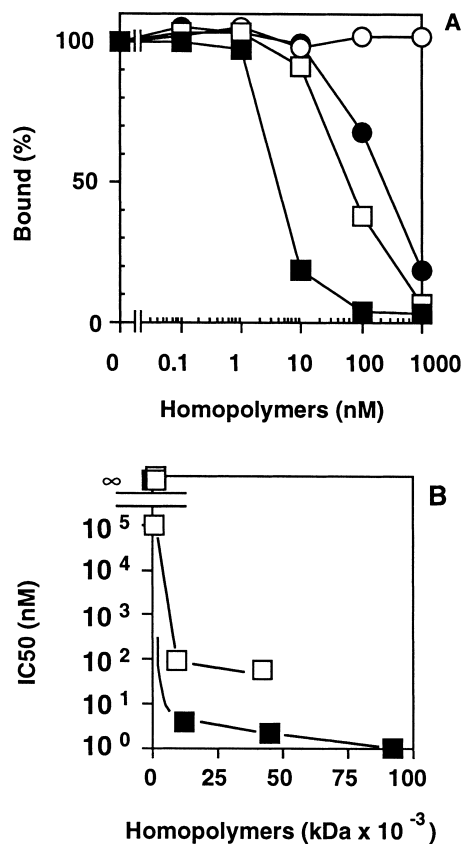


Fig. 3. Effect of homopolyamino acids on the interaction of 125 I-angiogenin with RI. Competition for binding of 125 I-angiogenin to RI by various synthetic polyamino acids. A: Effect of different homopolyamino acids. (■) Poly(Arg) (12.1 kDa); (□) poly(Lys) (9.6 kDa); (●) poly(Orn) (7.6 kDa); (○) poly(Asn) (10.4 kDa). B: Size effect of arginine and lysine polymers on the binding of 125 I-angiogenin to RI. Arginine, lysine and the following polymers were studied: poly(Arg): 12.1, 45.5 and 92.0 kDa; poly(Lys): 0.665, 1.0, 9.6 and 42.0 kDa. IC_{50} values were assimilated to the ∞ value for compounds whose competitive effect was below 50% at 100 μ M. Values are the means of duplicate determinations that differed by less than 5%.

as RI dissociated from the insolubilized proteins at higher salt concentrations. These results suggest that the activity of highly basic polypeptides is determined by their ability to bind to RI, thereby preventing the association of angiogenin with RI.

Table 1
Effect of histones and protamines on the binding of 125 I-angiogenin to RI

Protein	IC_{50} (μ M)	Total number of residues per mol	Basic residues (%)			Ref.
			Arg	Lys	Arg+Lys	
H2A	2.90	129	9.3	10.9	20.2	[31]
H2B	0.69	125	6.4	16.0	22.4	[32]
H3	0.70	135	13.3	9.6	23.0	[33]
H4	1.00	102	10.8	13.7	24.5	[34]
S1	0.59	87	35.6	9.2	44.8	[19]
HP3	0.25	54	48.1	3.7	51.8	[35]
Z3	0.37	37	64.8	0	64.8	[36]

RRIA was performed as described in Section 2. Proteins are listed according to their basic residue contents (%). The molecular masses of the proteins (in Da) were: H2A, 13 962; H2B, 13 777; H3, 15 257; H4, 11 238; S1, 11 179; HP3, 7260 and Z3, 4748. Values for IC_{50} are the means of duplicate determinations that differed by less than 5%.

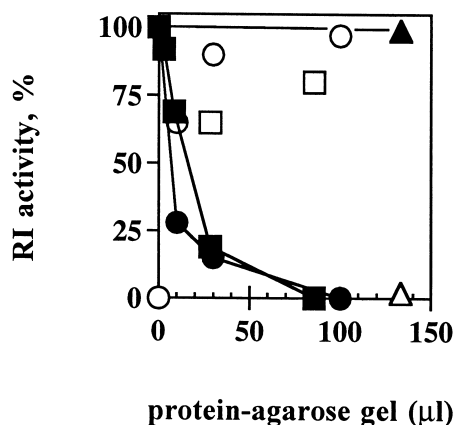


Fig. 4. Reversible interaction of RI with poly(Lys) and protamines. RI (0.12 U) was incubated with increasing volumes of poly(Lys)-agarose (■,□), protamine-agarose (●,○) and protein A-agarose (▲,△). Protein-agarose beads were centrifuged and supernatants were tested for their RI content using the RRIA (■,●,▲). RI activity released from the beads at a high salt concentration was also determined (□,○,△). Values are the means of duplicate determinations that differed by less than 5%.

4. Discussion

The mammalian RNase superfamily consists of related enzymes whose archetype is RNase A [1,2]. These RNases share common structural determinants, have conserved catalytic amino acid residues, and are thought to have evolved from a single ancestral gene [1]. Their catalytic properties differ in terms of kinetic constants and substrate specificities; several of these RNases, including pancreatic RNase, EDN, BS-RNase and tumor RNase, are potent enzymes while others, exemplified by angiogenin and ECP, have extremely low ribonucleolytic activity in conventional assays and were discovered through their biological properties (reviewed in [2]).

RI, a cytosolic protein, inhibits a number of these RNases with one to one stoichiometry [10,11]. Using a radio-ribonuclease inhibitor assay that detects interacting RNases, we focused on potential antagonists of angiogenin binding to RI. We observed that basic homopolypeptides interfered with this binding, the most potent inhibitor being poly(Arg). The inhibition mediated by homopolypeptides was likely dependent on their ionic charge as poly(Asn) had no effect. It was also relative to their size as arginine, lysine and lysine oligomers had no significant activity while higher molecular weight polymers were fully active. Similarly, several histones and protamines were potent inhibitors of angiogenin binding to RI. As poly(Lys) and protamines were shown to interact directly with RI, we suggest that the other basic competitors act similarly, therefore preventing angiogenin binding by steric hindrance. The effect of highly basic proteins may be related to the presence of five lysine and one arginine residues in RNase A (Lys-7, Lys-31, Lys-41, Lys-66, Lys-91 and Arg-39) that are involved in its binding to RI [12]. As regards angiogenin, apart from the involvement of Lys-40, six arginine residues (Arg-5, Arg-24, Arg-31, Arg-32, Arg-95 and Arg-121) substantially contribute to RI binding [13]. The strong involvement of basic residues in the binding of angiogenin to the inhibitor might be mimicked in part by basic homopolyamino acids, histones and protamines.

The biological function of RI is unknown. Present hypoth-

eses addressing this question are based on its high potency to inhibit RNases and suggest a regulatory and/or protective role of RI [11]. However, the physiological relevance of such interactions has not yet been proved, a major concern being that the inhibitor is an intracellular protein whereas RNases are secreted. The possibility that RI be endowed with distinct functions cannot be excluded as well. The striking sensitivity of RI to oxidative stresses in nucleated cells and erythrocytes [16,27] raises additional questions as to its role and metabolism. Research on the possible occurrence of RNase/angiogenin in intracellular compartments [28], associated with a better knowledge of the subcellular location of RI, will be necessary to approach these questions and those related to the significance of the binding of RI to highly basic proteins. Studies on the biological properties of RI should also be facilitated by the design of specific inhibitors. The extreme potency of poly(Arg) makes RI a preferential target *in vivo*, as higher concentrations of the synthetic peptide are usually required for modulation of other protein functions [29,30]. Starting from the basic structure of poly(Arg), heteropolyamino acids with tighter binding to RI could be synthesised, therefore opening new possibilities for such investigations.

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