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New muteins of RNase A with enhanced antitumor action

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Abstract Monomeric bovine pancreatic RNase A has been transformed into a dimeric ribonuclease with antitumor activity (Di Donato, A., Cafaro, V. and D'Alessio, G. (1994) J. Biol. Chem. 269, 17394–17396). This was accomplished by replacing the residues located in the RNase chain at positions 19, 28, 31, and 32, with proline, leucine, and two cysteine residues, respectively, i.e. those present at identical positions in the subunit of bovine seminal RNase, a dimeric RNase of the pancreatic-type superfamily, endowed with a powerful antitumor action. However, as an antitumor agent this mutant dimeric RNase A is not as powerful as seminal RNase. We report here site-directed mutagenesis experiments which have led to the identification of two other amino acid residues, glycine 38 and 111, whose substitution in the polypeptide chain of the first generation dimeric mutant of RNase A, is capable of conferring to the mutein the full cytotoxic activity characteristic of native seminal RNase.

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Key words: Ribonuclease; Antitumor; Protein engineering

1. Introduction

Protein engineering techniques, and site-directed mutagenesis in particular, are powerful tools for understating the relationships between the structure and the function of proteins. We have applied such an approach for investigating the molecular basis of the antitumor action of bovine seminal RNase (BS-RNase) [1].

BS-RNase is a dimeric RNase, homologous to pancreatic RNase A, with more than 80% sequence identity [2]. Two dimeric structures have been described for BS-RNase [3,4], which interconvert into each other, so that BS-RNase is an equilibrium mixture of two quaternary conformations [3]: one (named M×M) in which the subunits exchange their N-terminal helices, the other (named M = M) in which no exchange occurs. It should be added that BS-RNase is very active on double-stranded RNA, whereas most monomeric RNases have no activity under the same conditions [5].

The exact mechanism of the antitumor action of BS-RNase is not completely known. It has been determined [6] that the internalization of the protein by non-malignant cells has no apparent effect, whereas in malignant cells BS-RNase is found to degrade ribosomal RNA and inhibit protein synthesis. Furthermore, using a variety of experimental approaches we have discovered that BS-RNase cytotoxic action depends on its catalytical activity as a ribonuclease [7], and on its dimeric structure [7]. Its peculiar quaternary structure, in which the two subunits exchange their N-terminal helices is also important for a full antitumor activity [8].

By protein engineering techniques, we were able to transform RNase A, monomeric and devoid of the cytotoxic activity characteristic of BS-RNase, into an effective antitumor agent. This was accomplished [9,10] by substituting into its polypeptide chain four residues with those present at the same positions in BS-RNase: Pro, Leu, and two Cys, at positions 19, 28, 31, and 32, respectively. However, the antitumor activity of this mutein of RNase A is only about 60% that of native BS-RNase [9]. This indicates that other determinants must exist that are absent in the dimeric mutant of RNase A as constructed.

Recently, S. Benner and coworkers [11,12] have identified the main determinants of the activity of BS-RNase on double-stranded RNA. In particular, their results point to the important role of the Gly residues at positions 38 and 111. These residues are simultaneously present only in the subunit chain of seminal RNase, and in no other member of the terrestrial RNase superfamily ([13] and references therein).

Here we report that second generation dimeric mutants of RNase A, which include the additional substitutions D38G and E111G to the already known sequence determinants P19, L28, Cys-31 and Cys-32, possess an increased antitumor action, and that the full complement of the latter mutations is necessary to obtain a mutant of RNase A with a cytotoxic action as powerful as that of natural BS-RNase.

2. Materials and methods

2.1. DNA manipulations

Bacterial cultures, plasmid purifications and transformations were performed according to Sambrook et al. [14]. Double-stranded DNA was sequenced with the dideoxy method of Sanger [15], carried out with a Sequenase Sequencing Kit and labelled nucleotides from Amersham. The cDNa coding for PLCC-RNase A was prepared as described [9]. The mutations reported here were introduced in the PLCC-RNase A cDNA following the methodology of Kunkel [16] using mutagenic oligonucleotides synthesized at the Stazione Zoologica 'A. Dohrn' (Naples, Italy). The mutations were always verified by DNA sequencing. *E. coli* strain JM101 was from Boehringer; plasmid pET22b(+) and *E. coli* strain BL21(DE3) from AMS Biotechnology. Enzymes and other reagents for DNA manipulation were from Prom-

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Abbreviations: RNase A, bovine pancreatic ribonuclease A; BS-RNase, bovine seminal RNase; PLCC-RNase AA, (A19P, Q28L, K31C, S32C) dimeric RNase A; PLCC(G38)-RNase AA, (A19P, Q28L, K31C, S32C, D38G) dimeric RNase A; PLCC(G111)-RNase AA, (A19P, Q28L, K31C, S32C, E11G) dimeric RNase A; PLCCGG-RNase AA, (A19P, Q28L, K31C, S32C, G38, G111) dimeric RNase A; EDTA, ethylenediamine tetraacetic acid; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecylsulfate; Tris, tris(hydroxymethyl)aminomethane; DTT, dithiothreitol; IPTG, isopropyl-β-D-thiogalactopyranoside; MOPS, morpholinepropane sulfonic acid; Poly(A)·Poly(U), polyadenylic-polyuridylic acid (double-stranded); dsRNA, double stranded RNA

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ega Biotech. *Aeromonas proteolytica* aminopeptidase was purchased from Sigma.

2.2. Proteins

BS-RNase was purified from seminal vesicles as described by Tamburrini et al. [17].

The cloned cDNAs coding for the recombinant proteins, inserted into the pET22b(+) expression vector, were expressed and purified essentially as described previously [9,10]. Yields of dimers ranged from 8 to 10 mg per liter of bacterial culture. The percentage of M×M and M=M forms in dimeric mutant RNases was determined as previously described [3].

2.3. Antitumor assays

Cytotoxicity of proteins was assayed as described [7] on SVT2 cells (ATCC, Richmond, VA, USA), grown in Dulbecco's modified Eagle's medium, supplemented with 10% FCS, 4 mM glutamine, 400 U/ml penicillin and 0.1 mg/ml streptomycin. Cells were seeded in 24-well plates (2.5×10^4/well) in the presence of the protein to be tested. Cell survival, with respect to control cultures grown in the absence of the protein, was measured in triplicate. Controls on the absence of cytotoxicity of the RNases under study on the parent non-transformed 3T3 cells were routinely run.

2.4. Kinetic assays

RNase activity on yeast RNA was assayed with the method of Kunitz [18]. Poly(A)·Poly(U) was purchased from Sigma and used as a substrate [19] for the RNase muteins in 100 mM MOPS, pH 7.5, containing 100 mM NaCl. The specific activities were calculated from the initial rates by measuring the slope of the linear parts of the recordings, and were expressed as an increase in absorbance at 260 nm per minute per mg of protein with 40 μg/ml of Poly(A)·Poly(U) at 25°C.

2.5. Other methods

SDS-PAGE was carried out according to Laemmli [20]. Protein sequence determinations were performed on an Applied Biosystems sequencer mod. 473A, connected on-line with the HPLC apparatus for identification of phenylthiohydantoin derivatives. The electrospray mass spectrometric analyses were performed on a VG Bio Q mass spectrometer (VG Analytical) at CEINGE Biotecnologie Avanzate (Naples, Italy).

3. Results and discussion

The antitumor activity of the mutant PLCC-RNase AA is only about 60% that of native BS-RNase [9]. This mutant was obtained [9,10] by replacing into RNase A polypeptide chain the residues present at positions 19, 28, 31, and 32 with those present at the same positions in the subunit of BS-RNase: Pro, Leu, and two Cys, respectively.

We have previously demonstrated [21] that full antitumor action of recombinant seminal RNase depends on the removal of its N-terminal unprocessed methionine. Although neither crystallographic nor biological data point out any specific role of the N-terminal residue of the polypeptide chain of BS-RNase, we first explored the possibility that the methionine residue present at the N-terminal of the mutant PLCC-RNase AA could impair the full antitumor activity of the mutant. We thus removed the (−1) methionine of the recombinant PLCC-RNase AA, and assayed the mature protein for its cytotoxic activity. The results of the assays (data not shown) indicated that the removal of the N-terminal methionine does not affect the antitumor action of PLCC-RNase AA.

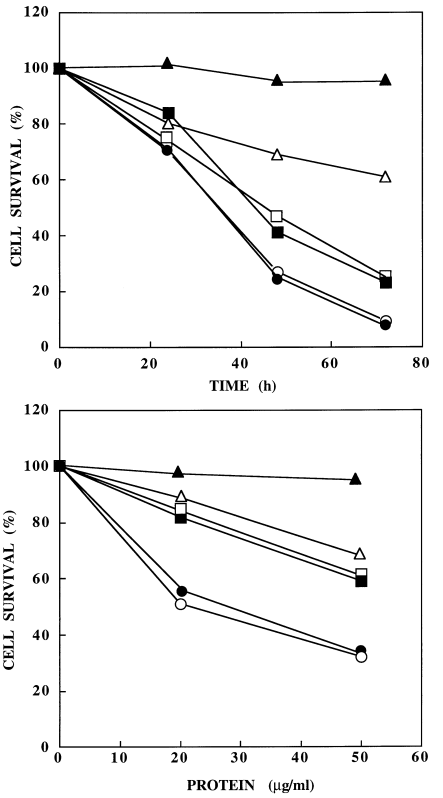

Fig. 1. Survival of malignant fibroblasts grown in the presence of (▲) RNase A, (○) BS-RNase, (△) PLCC-RNase AA, (□) PLCC(G38)-RNase AA, (■) PLCC(G111)-RNase AA, and (●) PLCCGG-RNase AA. A: Survival of cells grown in the presence of 50 μg/ml of each RNase; B: dose response curve determined after 48 h of growth.

Thus we turned our attention to another of the features essential for the antitumor action of BS-RNase: its catalytic activity as a ribonuclease [7]. The comparison of RNase A, inactive as cytotoxic agent, and BS-RNase, endowed with specific cytotoxicity towards tumor cells, with respect to their catalytic activity, has revealed that BS-RNase possesses catalytic activity towards double-stranded RNA [5], which is absent in RNase A assayed under the same conditions [5]. Recently, Benner and coworkers [22] have given evidence that Gly-111 and Gly-38 in BS-RNase account for the enhanced action of the enzyme against duplex nucleic acid structures.

Table 1
Catalytic activity and M×M content of the mutants of RNase A

Protein	M×M (%) at time 0	M×M (%) at 96 h	ssRNA[a]	Poly(A)·Poly(U)
RNase A			**100** ± 2	1.2 ± 0.1
PLCC-RNase AA	16 ± 1	71 ± 1	78 ± 4	3.50 ± 0.15
PLCC(G38)-RNase AA	58 ± 1	71 ± 1	75 ± 3	35 ± 3
PLCC(G111)-RNase AA	13 ± 1	70 ± 1	75 ± 4	8.0 ± 0.5
PLCCGG-RNase AA	62 ± 1	69 ± 1	54 ± 4	33 ± 2
BS-RNase	70 ± 2	70 ± 2	42.0 ± 1.5	11 ± 1

[a]Relative to RNase A specific activity, taken as **100**. The activity of RNase A ranged between 99 and 113 Kunitz units/mg of protein.

Thus, given the final target in the mechanism of the antitumor action of BS-RNase [6], i.e. ribosomal RNA, we prepared second generation dimeric mutants of RNase A which included, besides the already known sequence determinants P19, L28, Cys-31 and Cys-32, the additional substitutions D38G and E111G, in various combinations.

The mutant cDNAs, obtained as described in Section 2, were expressed in *Escherichia coli* as described previously for the mutant PLCC-RNase AA [10]. Briefly, the muteins, all found only in inclusion bodies, were first totally reduced and unfolded, and then refolded in the presence of a glutathione redox buffer into catalytically active monomers, linked through mixed disulfides with two glutathione moieties. The protecting glutathione moieties were then removed by mild reduction, followed by dialysis. Under these conditions, the monomers associated into homogeneous dimers linked by intersubunit disulfides, as indicated by SDS-PAGE analyses run under non-reducing and reducing conditions (data not shown). The muteins were also characterized by N-terminal sequencing, and by electrospray mass spectrometric analyses of their molecular weights.

Given the dependence of the antitumor activity of BS-RNase [8], and of the dimeric mutants of RNase A [10] on their quaternary conformation, i.e. on the presence of the more active M×M forms, we first measured the exchanging dimer content of the preparations of the mutants PLCC(G38)-, PLCC(G111)-, and PLCCGG-RNase AA (see Table 1), and then incubated each mutant for 96 h under conditions that induce the transformation of the M=M form into M×M.

Data in Table 1 show that at equilibrium the M×M content of all the mutants is similar to that of natural BS-RNase, and to that of parent PLCC-RNase AA. Thus, it seems that mutations D38G and E111G do not alter the thermodynamic barrier between non-exchanged (M=M) and exchanged (M×M) dimers. A further indication can be derived from the data collected measuring the M×M content of each protein preparation just after its purification. It is evident from the data in Table 1 that the content in the exchanging forms of both mutants harboring mutations D38G is much higher than that of the other mutant, almost approaching the equilibrium value. This would indicate a greater kinetic propensity of these mutants to displace the N-terminal helix from the body of one subunit to the other, which might depend either on the loss of a negative charge at position 38 (glycine replaces an aspartic acid residue at this position in both mutants) and/or on the loss of a bulky side chain, which is in the contact region between the exchanged N-terminal helix and the body of the other subunit [4].

The selective cytotoxic effect of these mutated ribonucleases was tested by measuring the percent of survival of SVT2 fibroblasts as derived from growth curves obtained in the absence or in the presence of 50 μg/ml of the proteins under test, and from a dose-response experiment (Fig. 1).

All dimeric mutants displayed a strong cytotoxic effect. The data lead to the conclusion that while wild-type RNase A has no effect on cell growth, and the antitumor effect of PLCC-RNase AA is about 60% of that of natural BS-RNase, the introduction of a glycine residue either at position 38 or at position 111 into PLCC-RNase AA polypeptide chain increases the antitumor action of the corresponding mutant, with respect to the parent protein. This effect is additive as the mutant with both glycine residues at positions 38 and 111 displays a cytotoxic effect, in fact as powerful as that of natural BS-RNase.

Benner and coworkers have recently found [22] that the ability of dimeric mutants of RNase A to cleave dsRNA depends on the presence of residues G38 and G111. They have also shown [12] that dimeric RNases joined by disulfide bridges between Cys residues 31 and 32 of both subunits, can be linked through a 'parallel connectivity' with Cys-31 and Cys-32 of one subunit linked with Cys-31′ and Cys-32′, respectively, of the other subunit, or by an 'antiparallel connectivity', with Cys-31 and Cys-32 of one subunit linked with Cys-32′ and Cys-31′, respectively, of the other subunit. In turn, the kind of connectivity influences the ability of the dimers to exchange their N-terminal helices, the dimers being linked through a 'parallel connectivity' impaired to displace their N-terminal segments [12].

We also assayed our mutants, differing from those used by Benner and coworkers, for having residue P19, for their ability to cleave single- and double-stranded structures.

The data reported in Table 1 show that the dimeric mutant PLCC-RNase AA, which includes all the determinants known to contribute to the dimeric structure and to the exchange properties of BS-RNase, has a reduced activity on single-stranded RNA with respect to parent monomeric RNase A, as it is typical of dimeric RNases, and an enhanced action, 3 times higher, on double-stranded RNA, with respect to monomeric RNase A. Also the glycine residue at position 111 contributes to the activity on dsRNA, as the activity of mutant PLCC(G111)-RNase AA is about 8 times higher, with respect to pancreatic RNase A. But the main contribution to the capability of degrading double-stranded substrates is given by glycine 38. In fact, mutant PLCC(G38)-RNase AA shows a 28-fold increase in the activity towards dsRNA, confirming the data reported by Opitz et al., although the activity values reported in the present paper are lower than those reported by Opitz [22]. These differences are most probably due to the lower ionic strength in our assay mixture. Moreover, it would seem that the contribution of residue G111 is rather marginal, as mutant PLCCGG-RNase AA displays an increase in the activity with respect to RNase A, almost identical to that of PLCC(G38)-RNase AA. It should also be noted that the activity of the double mutant on single-stranded RNA is more similar to that of BS-RNase, when compared to that of the other mutants. Thus, the data reported in the present paper, in agreement with those reported by Opitz [22], would indicate a key role of the residue present at position 38 in determining the strong increase in the catalytic activity of the dimeric mutants of RNase A on dsRNA, with respect to the parent protein. These results are in line with the proposal of Sorrentino et al. that the destabilization of polynucleotide secondary structures by RNases depends on the localization of a high positive charge density in discrete regions of the molecule, rather than on the global net positive charge of the molecule [19]. Thus, the contribution of the residue present at position 38 of mutants of RNase A might be another determinant of their high catalytic activity on dsRNA, besides the localized presence of positive charge density on the surface of the molecule.

Thus, our data indicate that the cytotoxic activity of the mutants of RNase does not seem to be related to the ability of the same mutants to degrade dsRNA. While both mutations

at positions 38 and 111, with a Gly residue replacing Asp and Glu, respectively, seem equally essential to the full display of antitumor action, the main contribution to the activity on dsRNA seems to derive from the presence of a Gly residue at position 38. Thus, a linear correlation between the activity on double-stranded RNA of the mutants of pancreatic RNase A harboring mutations D38G and E111G, and their cytotoxic activity cannot be made. However, the interplay between the presence of Gly residues at positions 38 and 111, and the connectivity between the dimers, and hence their tendency to exchange their N-terminal helices, could be the cause of the enhanced antitumor activity of the mutants carrying those residues. Alternatively, it could be hypothesized that the removal of two negative charges, that of aspartate 38 and glutamate 111, might favor the binding of the muteins to the extracellular matrix, and/or their transfer to cytosol through membranes. Both events have been implicated in the mechanism of the antitumor action of BS-RNase [6,23].

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