Biochimica et Biophysica Acta 1808 (2011) 3007–3015

Contents lists available at SciVerse ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamem

Enforcing the positive charge of N-termini enhances membrane interaction and antitumor activity of bovine seminal ribonuclease

Gerardino D'Errico[a,b], Carmine Ercole[a], Marisa Lista[a], Elio Pizzo[c], Annarita Falanga[d], Stefania Galdiero[d,e], Roberta Spadaccini[a,f], Delia Picone[a,*]

[a] Dipartimento di Chimica "Paolo Corradini", Università degli Studi di Napoli "Federico II", Via Cintia, 80126 Napoli, Italy
[b] CSGI, Consorzio interuniversitario per lo sviluppo dei Sistemi a Grande Interfase, Italy
[c] Dipartimento di Biologia Strutturale e Funzionale, Università degli Studi di Napoli "Federico II", Via Cintia, 80126 Napoli, Italy
[d] Dipartimento di Scienze Biologiche, Università degli Studi di Napoli "Federico II", Via Mezzocannone 16, 80134 Napoli, Italy
[e] Istituto di Biostrutture e Bioimmagini, CNR, Via Mezzocannone 16, 80134 Napoli, Italy
[f] Dipartimento di Scienze Biologiche ed Ambientali, Università del Sannio, Via Port'Arsa 11, 82100 Benevento, Italy

ARTICLE INFO

Article history:
Received 20 June 2011
Received in revised form 29 July 2011
Accepted 4 August 2011
Available online 10 August 2011

Keywords:
Membrane interaction
Cytotoxic ribonuclease
ESR
SPR

ABSTRACT

Binding to cell membrane, followed by translocation into the cytosol and RNA degradation, is a necessary requirement to convert a ribonuclease into a cytotoxin for malignant tumor cells. In this paper, we investigate the membrane binding attitude of bovine seminal ribonuclease (BS-RNase) and its variant G38K-BS-RNase, bearing an enforced cluster of positive charges at the N-termini surface. By using a combination of biophysical techniques, including CD, SPR and ESR, we find for the two proteins a common, two-step mechanism of interaction with synthetic liposomes, an initial binding to the bilayer surface, driven by electrostatic interactions, followed by a shallow penetration in the lipid core. Protein binding effectively perturbs lipid packing and dynamics. Remarkably, the higher G38K-BS-RNase membrane interacting capability well correlates with its increased cytotoxicity for tumor cells. Overall, these studies shed light on the mechanism of membrane binding and perturbation, proving definitely the importance of electrostatic interactions in the cytotoxic activity of BS-RNase, and provide a rational basis to design proteins with anticancer potential.

1. Introduction

The central role of RNA in the regulation of many vital processes, occurring under both physiological and pathological conditions, has emerged clearly over the past 10 years (for a very recent review see [1]. As a consequence, most ribonucleases have received a considerable interest for their association with health and diseases, including human cancer development [2], and are considered potential targets for drug design [3] because of their strong cytotoxic activity toward malignant tumor cells. One of the enzymes of this class, the amphibian Onconase, reached also clinical trials [4]. Different RNAs are degraded by these proteins, whose antitumor activity is critically dependent on a complex series of biochemical events, involving interactions with different cellular compartments. The crucial steps of this process include binding to negatively charged cell membrane, translocation into the cytosol via endocytosis [5] and RNA degradation. Furthermore, to fully exploit their antitumor potential, ribonucleases have to resist to protease attack and to evade Ribonuclease Inhibitor [6], a 50 kDa horse-shoe shaped protein that binds with very high affinity most monomeric ribonucleases and hampers their activity [7]. Previous structure-activity relationship studies allowed the identification of the crucial features that these enzymes have to fulfill to exhibit antitumor activity: i) a strong positive surface potential to allow membrane interaction; ii) a functional catalytic site and iii) a 3D structure able to evade Ribonuclease Inhibitor.

In principle, it is possible to increase the antitumor potentiality of ribonucleases by chemical modifications or protein engineering but until now this target remained essentially elusive. Enforcing the cytotoxicity by influencing the enzymatic activity is not straightforward;

Abbreviations: CD, circular dichroism; DLPG, dilauroyl phosphatidylglycerol; DMEM, Dulbecco's Modified Eagle's medium; DMPG, dimyristoyl phosphatidylglycerol; EIE, electrostatic interaction energy; ESR, electron spin resonance; LUVs, large unilamellar vesicles; MLVs, multilamellar vesicles; MTT, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; *n*-PCSL, spin-labeled phosphatidylcholines with the nitroxide group at different positions *n*, in the *sn*-2 acyl chain; PBS, phosphate buffered saline; SPR, surface plasmon resonance; SUVs, small unilamellar vesicles; T_m, melting temperature

* Corresponding author at: Dipartimento di Scienza degli Alimenti, Reggia di Portici-Via Università 100-80055, Portici (Na), Italy. Tel.: +39 081674406; fax: +39 081674499.
E-mail address: delia.picone@unina.it (D. Picone).

0005-2736/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbamem.2011.08.009

indeed, while the presence of a functional catalytic site is required, there is no direct correlation between catalytic efficiency and antitumor potentiality [8]. On the other hand, the enhancement of RI resistance is more feasible, since RNase A and human pancreatic ribonuclease variants designed to avoid binding were found to be cytotoxic [9,10]. Strategies to enhance ribonucleases ability to evade RI have been inspired by naturally occurring antitumor ribonucleases, which avoid binding because either they lack residues directly involved in the complex with RI, like in the case of Onconase, or their 3D structure does not allow the formation of a stable complex with RI. This has a paradigmatic example in bovine seminal ribonuclease (BS-RNase) [11], which shows antitumor activity *in vitro* and on model animals [12]. BS-RNase is a dimeric protein constituted by two identical subunits covalently linked through two disulfide bridges (for a review, see [7]); it is isolated as an equilibrium mixture of two isoforms, with or without exchange (swapping) of the N-termini, indicated as MxM and M = M respectively [13]. X-ray structures [14,15] revealed that the two isoforms present only minor differences in their tertiary structure, located essentially at level of 16–22 hinge region, *i.e.* the loop connecting the dislocating extremity to the protein body. However, under reducing conditions, the two isoforms behave differently: the swapped form, being converted into a so-called non-covalent dimer (NCD), still evades RI binding [16], whilst the unswapped one dissociates into two monomers, which are inactivated by RI. Thus, it could be hypothesized that an increasing of the MxM fraction with respect to M = M should result in an increased ability to evade RI and, consequently, in a higher antitumor activity. However, we have obtained enzymatically active BS-RNase variants with the same swapping amount of the native protein but a very low antitumor activity [17], suggesting that the swapping *per se* is not sufficient to elicit the antitumor activity, and making the relationships between swapping and cytotoxicity still complicated.

As last strategy to enhance ribonucleases antitumor activity one could act on their internalization efficiency, relying on the pieces of evidence that ribonucleases directly injected into the cytosol are more toxic than those added to cells externally [18]. Also in this case, natural ribonucleases are useful templates for protein engineering. A correlation with the number of positive charges on the protein surface and the cytotoxic activity has been already reported for several vertebrate ribonucleases, including Onconase [19], RNase A oligomers [20] and BS-RNase [21]. Particularly, in comparison with RNase A, the higher pI of BS-RNase enhances the interaction with phospholipid membranes and allows a better internalization [22]. In addition, based on *in silico* studies, it has been proposed that the BS-RNase face interacting with membrane is the one hosting the N-termini (henceforth designed N-face) which, in the native protein, is characterized by a large positive potential that promotes a strong interaction with the negatively charged phospholipids of the cellular membrane [21]. However, this hypothesis has not been experimentally proved so far.

Based on these premises, in this paper we have explored the possibility to strengthen the antitumor activity of BS-RNase by improving the protein/membrane through the enhancement of the positive potential of this same surface, *i.e.* we have produced the G38K-BS-RNase variant (henceforth called G38K), and found that it shows a higher cytotoxicity toward tumor cells. Since this variant does not present any change both in the catalytic activity and in the ability to evade RI, we hypothesized the higher cytotoxicity to be connected to its enhanced interaction with membranes. To support this idea, we performed a detailed investigation of the membrane interaction process of BS-RNase and G38K with synthetic liposomes by using a combination of biophysical techniques. These studies shed light on the mechanism of membrane interaction, proving definitely the importance of electrostatic interactions in the cytotoxic activity toward tumor cells, and can be helpful to design proteins with even improved antitumor potentiality.

2. Materials and methods

2.1. Homology modeling and electrostatic potential calculation

The homology model of the G38K variant was calculated upon the crystal structure of the swapped BS-RNase (pdb code 1BSR) using Modeller 8v5 program [23]. The quality of the structural models was evaluated with the same program using the score of variable target function method [24]. Model analyses were performed using MOLMOL [25] and PyMOL [26]. The electrostatic potential was calculated using the program MGLTools-PMV.

2.2. Determination of electrostatic energy of interaction with charged membranes

The orientation-dependent electrostatic free energy due to the presence of a uniform electrostatic field generated by a charged membrane was computed by solving the Poisson-Boltzmann equation using the program DELPHI4 [27]. Similar to the modeling study of Notomista et al. [21] we assumed a surface charge density of 192 negative charges distributed over an area of 117×108 Å^2, which results in a strong uniform electrostatic field of 0.352×10^9 V/m in the direction of the z-axis. The dimer was placed at a distance of 55 Å from the membrane, in order to avoid close contacts which are difficult to describe with a continuum model. The molecule was then rotated in steps of 30° around the z-axis. For each rotation a second rotation around the x-axis in steps of 30° was applied. A third rotation around the z-axis should be applied to complete the rotational space sampling. This was not done because of the symmetry of the applied electric field.

2.3. Protein samples

The QuikChange Site-Directed Mutagenesis Kit protocol (Stratagene, La Jolla, CA) was used to substitute glycine 38 with lysine (G38K) in pET-22b(+) plasmid cDNA coding for BS-RNase, already available in our laboratory, which contained the N67D substitution to avoid local heterogeneity arising from the spontaneous deamidation occurring at position 67 [28,29].

The monomeric derivatives of BS-RNase and its G38K variant were expressed in *E. coli* cells and purified as previously described [30,31], with cysteines 31 and 32 linked to two glutathione molecules. Once assessed the purity of the protein samples and the correctness of their fold by gel-electrophoresis, CD and 2D NMR analysis, the enzymatic activity on yeast RNA was evaluated as usual [32] and found very close to each other for the two proteins, and also close to the literature data. The monomers were converted in the corresponding dimers following the procedure already reported [33]; as usual, the main product at the first purification step was the unswapped dimer. The protein solutions were then incubated at 37 °C to allow the conformational equilibrium between swapped and unswapped isoforms; the swapping extent was then assessed by two independent methods, as reported before [34], and found about 70% for both the recombinant proteins, as in native BS-RNase. Also for the dimeric form of G38K the enzymatic activity on yeast RNA was found very close to that of the parent BS-RNase.

2.4. Cytotoxicity studies

Before the cytotoxicity assays, the N-terminal methionine was removed with *Aeromonas proteolytica* aminopeptidase (Sigma) [35].

Cytotoxicity of BS-RNase and G38K was evaluated by performing the 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction inhibition assay [36]. Simian-virus-40-transformed mouse fibroblasts (SVT2 cells) and the parental non transformed Balb/C 3T3-line (3T3 cells) were obtained from the A.T.C.C. (American Type Culture