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Binding of a type 1 RIP and of its chimeric variant to phospholipid bilayers: evidence for a link between cytotoxicity and protein/membrane interactions

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ABSTRACT

Ribosome-inactivating proteins (RIPs) are enzymes, almost all identified in plants, able to kill cells by depurination of rRNAs. Recently, in order to improve resistance to proteolysis of a type 1 RIP (PD-L4), we produced a recombinant chimera combining it with a wheat protease inhibitor (WSCI). Resulting chimeric construct, named PD-L4UWSCI, in addition to present the functions of the two domains, shows also an enhanced cytotoxic action on murine cancer cells when compared to PD-L4. Since different ways of interaction of proteins with membranes imply different resulting effects on cells, in this study we investigate conformational stability of PD-L4 and PD-L4UWSCI and their interaction with membrane models (liposomes). Circular dichroism analysis and differential scanning calorimetry measurements indicate that PD-L4 and PD-L4UWSCI present high and similar conformational stability, whereas analysis of their binding to liposomes, obtained by isothermal titration calorimetry and differential scanning calorimetry, clearly indicate that chimera is able to interact with biomembranes more effectively.

Overall, our data point out that WSCI domain, probably because of its flexibility in solution, enhances the chimeric protein interaction with membrane lipid surfaces without however destabilizing the overall protein structure. Analysis of interactions between RIPs or RIP based conjugates and lipid surfaces could provide novel insights in the search of more effective selective membrane therapeutics.

1. Introduction

The cancer is one of the leading causes of death in the world, in fact more than 14% of human deaths are imputable to this disease [1,2]. In this framework, the global scientific research is focused on developing therapeutic strategies to obtain a successful treatment of cancer, despite the serious difficulties due to the emergence of resistance, which often leads to the development of new abnormal cells with aggressive characteristics [3]. During the years, cancer treatment has pursued various strategies able to specifically destroy cancer cells such as chemotherapy [4], immunotherapy [5] radiation therapy [6] and approaches by using immunotoxins [3,7] or treatments with hormones [8,9].

Among these strategies, the development of immunoconjugates involves the use of chimeric molecules obtained combining antibody or its portion and specific toxins that rely on intracellular toxin action to kill target cells [10]. This approach is based on antibody specificity to select target on cancerous cells, thus allowing an effective toxin delivery specifically on abnormal cells [11]. Generally, the toxin belong to enzymes, such as ribonucleases (e.g. RNase 1 [12] and α-sarcin [13]), ADP-ribosyltransferases (e.g. diphtheria toxin and exotoxin A from *Pseudomonas* [14,15]) or several *N*-glycosilases (e.g. ribosome inactivating proteins - RIPs) [16,17]. In all cases, many factors determine sensitivity or resistances to the immunoconjugates such as access to target cells, binding to target cells, cell entry, routing [18] or proteolysis susceptibility. The latter statement is well documented by several authors that described studies of RIPs based immunotoxins [19,20].

RIP enzymes (EC: 3.2.2.22) are a family of well-characterized toxins, which possess N-β-glycosilase activity able to cleave a specific

Abbreviations: (RIPs), Ribosome-inactivating proteins; POPC, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine; POPG, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoglycerol; DPPC, 1,2-dipalmitoyl-sn-glycero-3-phosphocholine; DPPG, 1,2-dipalmitoyl-sn-glycero-3-phosphoglycerol; MLVs, Multi-Lamellar Vesicles; SUVs, Small Uni-Lamellar vesicles; LUVs, Large Uni-Lamellar vesicles

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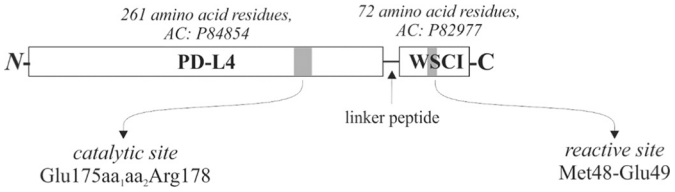
adenine residue from 28S rRNA in the exposed Sarcin/Ricin Loop (SRL) [21] and irreversibly to inhibit protein synthesis in eukaryotic cells [22]. RIPs are classified depending on if possess or not quaternary structure: type 1 basic proteins with N-glycosidase activity (A chain) without lectin-like chain (B chain) and type 2 chain with a lectin-like B chain linked to A chain by a disulphide bond [22,23]. Most of them are produced by plants [24] and are thought to represent a defence mechanism against viral or parasitic attackers [25–27].

Most effective bioactive conjugated are based on chemical linkage between RIP and antibodies, lectins, growth factors or nanoparticles [16,27]. However, action of RIPs, alone or as conjugates is closer correlated to its intracellular routing [28,29].

An increasing number of reports indicate RIPs as effective moiety in the construction of conjugates for developing selective antiviral and anticancer agents [17]. Cytotoxicity of RIPs, alone or as conjugates, is due not only to their enzymatic activity but also to their cellular routing. This is also confirmed by the existence of non-toxic RIPs (e.g. from *Sambucus*), that although retaining their N-glycosidase activity, are unable to kill the cells because they are involved in different intracellular routing with respect to toxic RIPs [30].

Our research group, in order to develop a new generation of RIP based chimeric conjugates less susceptible to proteolytic degradation during their intracellular routing, have designed a novel specific chimeric toxin [31]. It is composed, as below schematically reported, by a type 1 RIP from *Phytolacca dioica* L. (PD-L4; UniProt code: P84854) [32] and a protease inhibitor from endosperm of hexaploid seeds of *Triticum aestivum* L. (WSCI; UniProt code: P82977) [33].



The chimeric protein, named PD-L4UWSCI, consists of two domains: an amino-terminal PD-L4 domain fused to a C-terminal WSCI domain. Catalytic site of PD-L4 and reactive site of WSCI are indicated in grey. The linker peptide is composed by residues **LEQASSYTAPQPQPG**. AC: UniProt (http://www.uniprot.org/) accession number.

This recombinant construct, hereafter PD-L4UWSCI, has proved to possess intact intrinsic activity of both domains (e.g. enzymatic activity and inhibitory properties) and at the same time it presented an enhanced intriguingly selective cytotoxicity on murine tumour cells [31]. Subsequently, in order to verify if this increased selective cytotoxicity could be due to the anti-chimotryptic inhibitory specificity mediated by WSCI moiety, a mutant chimeric construct was produced, in which inhibitory specificity was modified (anti-chimotryptic versus anti-tryptic), substituting two amino acid residues (325[P1] and 326[P1']) [34]. As reported in [34], mutated specificity of inhibition on WSCI domain, did not alter cytotoxicity of chimeric construct. This invalidate the hypothesis on a possible contribution of WSCI specificity to anti-tumour action of the chimeric construct but, at the same time it is conceivable that WSCI could contribute to the observed chimeric protein cytotoxic activity on malignant cells by altering structural/energetic properties of the protein and of its interaction capability with other biomolecules. Based on these considerations, it is plausible to imagine different ways of interaction between PD-L4 or PD-L4UWSCI, with membrane phospholipid bilayers; besides, it should be noted that normal and cancerous cells present different lipid compositions, which can play a key role in cell/macromolecules interactions [35–37].

In the present work, we characterized the conformational stability (by thermal and GuHCl-induced unfolding measurements) as well as the interaction with cancer cell membrane models, as of recombinant protein PD-L4 compared to the construct PD-L4UWSCI. Our data reveals that WSCI has a marginal effect on the protein stability but significantly increases the protein ability to interact with lipid bilayers and to modify the membrane biophysical properties.

2. Material and methods

2.1. Materials

Expression vector pET 22b($^+$) and *E. coli* strain BL21 (DE3) were from AMS Biotechnology (Lugano, Switzerland). Other reagents for DNA manipulation and rabbit reticulocyte lysate were from Promega Biotech (Milan, Italy) or were described elsewhere [31,34]. Trypsin, alpha-chymotrypsin, protease substrates (BTEE, NBA and TAME), antibiotics, DEPC, and reagents for cell culture were purchased from Sigma Aldrich (Milan, Italy). Bicinchoninic acid (BCA) kit was purchased from Pierce (Rockford, IL, USA).

2.2. Expression and purification of recombinant chimera or PD-L4

Expression, refolding, and purification of recombinant proteins (rPD-L4 or rPD-L4UWSCI) were performed according to Del Vecchio Blanco et al. [38] (**Fig. S1**). Each step of the expression and purification procedure was monitored by SDS-PAGE analyses. The eluted protein from the cation exchange chromatography on SOURCE 15S 4.6/100 PE column (1.7 mL) using Akta purifier system (GE Healthcare, Milan, Italy) was collected, dialyzed, and kept frozen until use.

2.3. Enzymatic assays

28S rRNA N-glycosidase activity was assayed as previously described [39] by incubating rabbit reticulocyte lysate (80 μL) with 2 μg of PD-L4 or PD-L4UWSCI. α-chymotryptic and anti-α-chymotryptic activities, as well as tryptic and anti-tryptic activities were determined as previously reported [33] by using BTEE and TAME as synthetic substrates.

2.4. Liposome preparation

Appropriate amounts of lipids were weighed and dissolved in chloroform/methanol (2/1 v/v). A thin film of the lipids was produced by evaporating the solvent with dry nitrogen gas, and placed in a vacuum overnight. The sample was then hydrated with a definite amount of 20 mM sodium phosphate buffer at pH 7.4 and vortexed obtaining a suspension of multilamellar vesicles (MLVs). Vesicles composed by DPPC/DPPG (80/20 mol%) were used for DSC measurements while vesicles composed by POPC/POPG (80/20 mol%) were used for ITC binding measurements. In the latter case, large unilamellar vesicles, (LUVs) were prepared by the extrusion methods using a Mini-Extruder (Avanti Polar Lipid Inc.). The liposome suspensions were extruded through 100 nm polycarbonate filters.

2.5. Circular dichroism

CD spectra in the far UV-region (190–250 nm) were recorded on a Jasco J-715 spectropolarimeter under constant nitrogen flow, equipped with a Peltier type temperature control system (Model PTC-348WI). The spectra were recorded in a 0.1 cm cell, with a 4 s response time, 2 nm bandwidth and 20 nm/min scan rate, averaged over three scans, and finally corrected for the buffer signal. Cell cuvette thickness and protein concentration were chosen in a way that the maximum high-tension voltage of the photomultiplier was not exceeding 600 V at the lowest wavelength of 190 nm. Mean residue ellipticity values (MRE), [θ] in deg cm^2 $dmol^{-1}$, were calculated by the following relation: $[\theta] = [\theta]_{obs} M_w / n\ 10 \times l \times C$, where θ is the measured ellipticity (millidegrees), M_w is the molecular mass of the protein in Da, n is the number of amino acid residues, C is the protein concentration in mg/mL, and l is the path length in cm. Typically, we used protein

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