Biochimica et Biophysica Acta 1811 (2011) 1038–1044

Contents lists available at SciVerse ScienceDirect

Biochimica et Biophysica Acta

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

Cholesterol and membrane phospholipid compositions modulate the leakage capacity of the swaposin domain from a potato aspartic protease (*St*Asp-PSI)

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ARTICLE INFO

Article history:
Received 7 March 2011
Received in revised form 8 July 2011
Accepted 3 August 2011
Available online 26 August 2011

Keywords:
Antimicrobial protein
Antitumor protein
Plant aspartic protease
Membrane destabilization
α-helix protein
Plat defense response

ABSTRACT

Potato aspartic proteases (*St*APs) and their swaposin domain (*St*Asp-PSI) are proteins with cytotoxic activity which involves plasma membrane destabilization. The ability of these proteins to produce cell death varies with the cellular type. Therefore, *St*APs and *St*Asp-PSI selective cytotoxicity could be attributed to the different membrane lipid compositions of target cells. In this work we investigate the possible mechanism by which *St*APs and *St*Asp-PSI produce selective membrane destabilization. Results obtained from leakage assays show that *St*Asp-PSI is a potent inducer of the leakage of LUVs containing anionic phospholipids, especially those containing phosphatidylglycerol. Based in these results, we suggest that the cytotoxic activity of *St*Asp-PSI on pathogenic microorganisms could be mediated by the attraction between the exposed positive domains of *St*Asp-PSI and the negatively charged microorganism membrane. On the other hand, our circular dichroism spectroscopic measurements and analysis by size exclusion chromatography and followed by electrophoresis, indicate that hydrophobic environment is necessary to *St*Asp-PSI oligomerization and both *St*Asp-PSI disulfide bounds and membrane with negative charged phospholipids are required by *St*Asp-PSI to produce membrane destabilization and then induce cell death in tumors and microorganism cell targets. Additionally, we demonstrate that the presence of cholesterol into the LUV membranes strongly diminishes the capacity of *St*Asp-PSI to produce leakage. This result suggests that the lack of hemolytic and cytotoxic activities on human lymphocytes of *St*Asp-PSI/*St*APs may be partly due by the presence of cholesterol in these cell membrane types.

1. Introduction

Aspartic proteases (EC 3.4.23) (AP) are a class of widely distributed proteases present in animals, microbes, viruses and plants [1,2]. Biological functions of plant APs have not yet been characterized to the extent of their mammalian, microbial or viral counterparts [1–4]. Most of plant AP sequences predict preproproteins, as in the case of animal and fungal aspartic proteases, with a signal peptide and a proregion at the amino-terminus of the mature protein [5]. However, plant AP genes have an extra region of approximately 100 amino acids known as "plant specific insert" (PSI) [3,4], similar to saposin-*like* proteins (SAPLIP) (Fig. 1A). Structural analysis of PSI domains reveals a compact globular structure formed by 5 alpha-helices linked to each other by disulfide bridges [4,6]. PSI is not a true saposin domain; it is the swap of the N- and C-terminal portions of the saposin like domain; hence, PSI is named as swaposin domain [4] (Fig. 1B).

Saposin and saposin-*like* proteins and domains have diverse *in vivo* biological functions or are implicated in different physiological functions [7]. All these proteins bind to or interact with lipid membranes and its "saposin-fold" is a common fold in a single globular structure [6–12] (Fig. 1C). Despite the conserved structural organization of SAPLIPs, their distinct modes of interaction with biological membranes are not fully understood. This could be the result of the differential interactions with the biological membrane environments and/or differences in lipid cell membrane composition [12–16]. Several functions have been proposed for PSI domain, as the targeting to the vacuole; vesicle leakage and a role in the processing of the mature enzyme [4,14,17]; however, their role(s) in the plants is/are still speculative.

Previously, we have reported the cloned, heterologous expression and purification of the PSI domain from a *Solanum tuberosum* aspartic protease (*St*Asp-PSI) [6]. *St*Asp-PSI has high structural similarity with

Abbreviations: AP, aspartic proteases; CD, circular dichroism; CF, 5-Carboxyfluorescein; Chol, Cholesterol; DTT, dithiothreitol; EPA, egg phosphatidic acid; EPC, egg L-α-phosphatidylcholine; EPG, egg L-α-phosphatidylglycerol; LUV, large unilamellar vesicles; MLV, multilamellar vesicles; PCD, programmed cell death; PSI, plant specific insert; SAPLIP, saposin-like proteins; *St*AP, *Solanum tuberosum* aspartic proteases; SUV, small unilamellar vesicles; TFE, 2,2,2-trifluoroethanol; TPE, egg trans-esterified L-α-phosphatidylethanolamine

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doi:10.1016/j.bbalip.2011.08.013

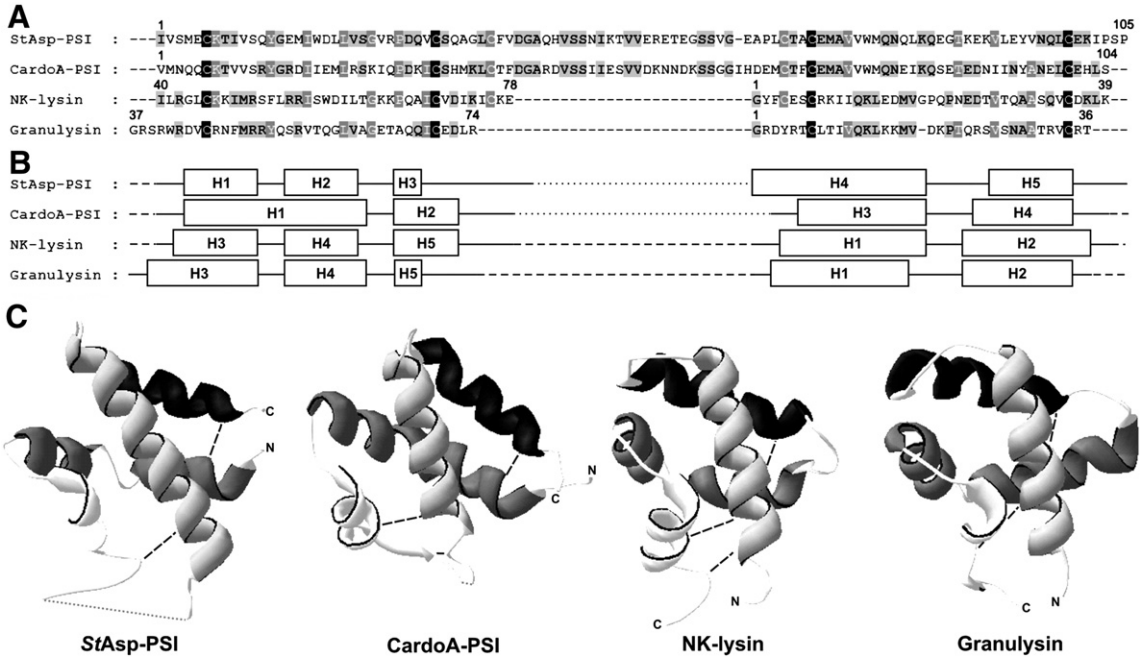

Fig. 1. Sequence and structural homology between *St*Asp-PSI, CardoA-PSI and SAPLIPs. (A) Amino acid sequence alignment of the PSI domain of *St*Asp-PSI (GeneBank accession no. AY672651) and CardoAPSI (GeneBank accession no. AJ132884) and permuted sequences of NK-lysin (GeneBank accession no. Q29075) and Granulysin (GeneBank accession no. EAW99485). Sequences retrieved from databases were automatically aligned using CLUSTAL W algorithm. Conserved cysteines are highlighted in black, identical residues are highlighted in gray and gaps are indicated by dashes. (B) The α-helical regions are indicated by boxes. The disordered part of the PSIs is marked by dotted line and gaps are indicated by dashes. (**C**) Ribbon representation of the model structure of the PSI domain of *St*Asp-PSI and CardoAPSI based on the crystal structure of prophytepsin-PSI (PDB accession no. 1QDM) and crystal structures of NK-lysin and Granulysin. Similar helixes are displayed in same color. The disordered part of the PSIs is marked by dotted line and disulfide bridges by dashed lines. Similarities between *St*Asp-PSI, CardoAPSI, NK-lysin (PDB: 1NKL) and Granulysin (PDB: 1L9L) were determined using DeepView/Swiss-PdbViewer 3.7 program.

two saposin-*like* proteins with antimicrobial activity, NK-lysin and granulysin [6,7]. Like these SAPLIPs, the *St*Asp-PSI domain is able to interact with cell plasma membranes, increasing the cell permeability and finally, producing cell death [6,18]. Additionally, in previous reports we demonstrated that the cytotoxic activity of *St*APs and *St*Asp-PSI is selective. Whereas these proteins are toxic to plant and human pathogen microorganisms and cancer cells, they are not able to kill human T cells, human red blood cells and plant cells [6,18–23]. Like with a vast number of antimicrobial peptides from various sources, *St*APs and *St*Asp-PSI have been isolated and characterized; however, the molecular explanation for their target specificity, or conversely, the reason of the different susceptibilities of cells from different origins to these proteins is not yet understood [6,18–24].

In order to search for the possible reasons of the differential cytotoxic activity of *St*Asp-PSI and *St*APs, we have analyzed in this work the ability of *St*Asp-PSI to induce membrane rupture of LUVs with different phospholipid compositions as well as changes in the *St*Asp-PSI secondary structure and oligomerization capacity in membrane like environments.

2. Materials and methods

2.1. Protein expression and purification

Recombinant *St*Asp-PSI was overexpressed in *Escherichia coli* M15 and purified as described previously [6]. Protein was stored in 50 mM phosphate buffer, 25 mM NaCl, pH 7. Protein concentration was measured by the bicinchoninic acid method [25], using BSA as the standard.

2.2. Materials and reagents

Cholesterol (Chol), total bovine liver lipid extract (liver extract), egg phosphatidic acid (EPA), egg L-α-phosphatidylcholine (EPC), egg L-α-phosphatidylglycerol (EPG), and egg trans-esterified L-α-phosphatidylethanolamine (TPE) were obtained from Avanti Polar Lipids (Alabaster, AL, USA). Calcein, 5-Carboxyfluorescein (CF, >95% by HPLC), dithiothreitol (DTT), 2,2,2-trifluoroethanol (TFE), Triton X-100 and EDTA were purchased from Sigma-Aldrich (Madrid, ES). All other chemicals were commercial samples of the highest purity available (Sigma-Aldrich, Madrid, ES). Water was deionized, twice-distilled and passed through a Milli-Q equipment (Millipore Ibérica, Madrid, ES) to a resistivity higher than 18 MΩ cm.

2.3. Vesicle preparation

Aliquots containing the appropriate amount of lipid in chloroform-methanol (2:1 vol/vol) were placed in a test tube, the solvents were removed by evaporation under stream of O_2-free nitrogen, and finally, traces of solvents were eliminated under vacuum in the dark for>3 h. The lipid films were resuspended in an appropriate buffer and incubated either at 25 °C or 10 °C above the phase transition temperature (T_m) with intermittent vortexing for 30 min to hydrate the samples and obtain multilamellar vesicles (MLV). The samples were frozen and thawed five times to ensure complete homogenization and maximization of protein/lipid contacts with occasional vortexing. Large unilamellar vesicles (LUV) with a mean diameter of 0.1 μm were prepared from MLV by the extrusion method [26] using polycarbonate filters with a pore size of 0.1 μm (Nucleopore Corp., Cambridge, CA, USA). Small unilamellar vesicles (SUV) were prepared from MLVs using a Branson 250 sonifier (40 W) equipped with a microtip until the suspension became completely transparent. Every 30 s, the samples were cooled for 90 s in ice to prevent overheating of the solution. The titanium particles from the tip were removed by centrifugation at 15000 rpm at room temperature for 15 min. For circular dichroism (CD) spectroscopy, SUVs of different lipid composition were mixed with *St*Asp-PSI to a protein/lipid ratio 1:25. The phospholipid concentration was measured as described previously [27].