Plant Physiology and Biochemistry 128 (2018) 178–184

Contents lists available at ScienceDirect

Plant Physiology and Biochemistry

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

Research article

Identification of novel phosphatidic acid binding domain on sphingosine kinase 1 of *Arabidopsis thaliana*

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ARTICLE INFO

Keywords:
Abiotic stress
ABA
Phosphatidic acid
Phospholipase D
Sphingosine kinase
Lipid binding domain

ABSTRACT

Phosphatidic acid (PA) is an important lipid signaling molecule which interacts with *Arabidopsis thaliana* Sphingosine kinase1 (AtSPHK1) during several abiotic stresses particularly drought stress as a result of Abscisic acid (ABA) signaling in guard cells. PA molecules respond by generating lipid signal and/or by binding and translocating target proteins to membrane. However, site of interaction and role of PA binding to AtSPHK1 is not clear yet. Owing to the importance of AtSPHK1 during stress signaling it is imperative to decipher the site of PA interaction with AtSPHK1. To identify the PA binding region of AtSPHK1, various deletion fragments from N-terminal and C-terminal region were prepared. Results from protein lipid overlay assay using various truncated proteins of AtSPHK1 suggested the involvement of N-terminal region, between 110 and 205 amino acids, in binding with PA. *In-silico* analyses performed to build homologous structure of AtSPHK1 revealed that PA docking occurs in the hydrophobic cavity of DAG-Kinase domain. Deletion of amino acids ^{182}VSGDGI^{187} perturbed PA-AtSPHK1 binding, indicating an essential role of these six amino acids in PA-AtSPHK1 binding.

1. Introduction

Plants constantly come across diverse biotic and abiotic stresses; thus have evolved complex mechanisms to perceive external signals that allow them to withstand in response to changing environmental conditions (Okazaki and Saito, 2014). Abiotic stresses include numerous stresses caused by complex environmental conditions, viz. salinity, strong light, drought, high and low temperatures, freezing, heavy metals and hypoxia (Hirayama and Shinozaki, 2010). These extracellular signals trigger various molecular mechanisms that converge into specific intracellular responses playing a vital role in plant stress response. Lipids are the basic constituents of the cell membrane, which provide structural basis and an energy stock for metabolism to the cell. It is also reported that lipids are mediators of several plant processes such as membrane trafficking, cytoskeleton rearrangements and signal transduction, which are essential for growth, differentiation and cell survival (Wang, 2004). Plants use a wide range of lipids such as glycerolipids, sphingolipids, free fatty acids, oxylipins, sterols, phosphatidylinositol, certain lyso-phospholipids, diacylglycerol (DAG) and phosphatidic acid (PA) (Farmer et al., 2003; Lindsey et al., 2003; Sperling and Heinz, 2003; Wang, 2004). In the response of specific biotic and abiotic factors, the production of lipid mediators is regulated by various lipid-signaling enzymes such as phospholipases, lipid kinases and phosphatases (Beisson et al., 2003; Holk et al., 2002; Kato et al., 2002; Lee et al., 2003; Mueller-Roeber and Pical, 2002; Nishiura et al., 2000; Pierrugues et al., 2001; Wang et al., 1994). Sphingolipids/long chain bases (LCBs) and ceramides have emerged as important mediators during signaling events in plants (Lynch et al., 2009; Pata et al., 2010).

Abscisic acid (ABA) is an endogenous phytohormone which increases during stress responses (Hubbard et al., 2010; Raghavendra et al., 2010). Several studies have reported that ABA regulates the developmental and stress signaling pathways (Hirayama and Shinozaki, 2007; Seki et al., 2002; Ton et al., 2009). PA is an important secondary messenger involved in the regulation of multiple mediators that regulate stomatal aperture in response to drought stress or due to action of related phytohormone ABA (Mishra, 2006). Various environmental and developmental responses such as nutrient starvation, low temperature, pathogen elicitation, freezing, wounding, drought, salinity, nodule induction and oxidative stress cause a sudden burst of PA (Guo et al., 2011). In plants, it has been observed that the local concentration of PA increases after perceiving stress signals, thus indicating a primary role of PA in stress response. PA is reported to regulate various cellular functions by effectively interacting with targeted proteins such as ABI1, PP2C phosphatase, phosphoenolpyruvate carboxylase (PEPC), CTR1 protein, NADPH oxidase, MAPK6 and Sphingosine kinase (SPHK) involved in abiotic stress (Testerink et al., 2004). Although more than 20

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https://doi.org/10.1016/j.plaphy.2018.04.039
Received 21 December 2017; Received in revised form 25 April 2018; Accepted 27 April 2018
Available online 08 May 2018
0981-9428/

plant proteins are known to interact with PA, (Testerink and Munnik, 2005), but a signature interacting motif/domain is still not identified. Several lipid binding domains such as Phox (PX) (Karathanassis et al., 2002), Pleckstrin homology (PH) (Deak et al., 1999) and C2 have also shown the affinity for PA (Lindsay, 2004) in some proteins. The interaction between PA and proteins requires specific conformational change which is unknown. PA has been suggested to tether the Sphingosine kinase in cytosol which leads to its recruitment towards the membrane (Guo et al., 2011). PA has fundamentally different roles in membrane dynamics and cell signaling.

Sphingosine Kinase (SPHK1) is a member of the DAG kinase family (Strub et al., 2010) and phosphorylates LCBs to LCBPs such as phyto-S1P in plants (Coursol, 2005). As reported, SPHKs in mammalian cell regulates many cellular processes including suppression of apoptosis, cell growth and pathophysiology of various diseases (Strub et al., 2010; Worrall et al., 2003). The SPHK phosphorylates the sphingolipids and forms the Sphingosine-1-Phosphate (S1P) which is further involved in different processes such as cell proliferation and survival in animals and promotes closing of open stomata in response to drought stress in plants (Coursol, 2005; Spiegel and Milstien, 2003).

Structural study of AtSPHK1 is important to understand the physiological role of PA to AtSPHK1 interaction. This study identifies the critical amino acids involved in the binding of PA to AtSPHK1. Seven different fragments from N-terminal region and C-terminal region of AtSPHK1 were cloned and purified. In addition, 3D homology model of AtSPHK1 was docked with PA and ADP to predict the site of interactions between protein and ligands. Molecular dynamics simulation was carried out to check the stability and affinity of PA with AtSPHK1. Further, protein lipid overlay assay and isothermal titration calorimetry were performed to determine the role of VSGDGI motif for the binding of PA with AtSPHK1.

2. Materials and methods

2.1. Cloning, expression and affinity purification of deletion fragments

The cDNA of *A. thaliana* Sphingosine kinase1 (*AtSphK1; At4g21540*) was amplified using primers SphK1-f and SphK1-r (1-485) and cloned by following the protocol of Zhang et al. (2004). Four fragments with C terminal deletion and three with N-terminal deletion were amplified by using cDNA as template. Deletion mutants were amplified using following primer pairs: SphK1-f-r4 (1-142), SphK1-f-r5 (1-172), SphK1-f-r2 (1-213), SphK1-f-r3 (1-316), SphK1-f1-r (110-485), SphK1-f2-r (206-485) and SphK1-f4-r (241-485) (Table 1 and Fig. 1A). The PCR reaction mixture consisted of 5X reaction buffer (10 μL), 2.5 mM dNTP mix (1 μL), 10 pmol/μL of each primer (1 μL), 100 ng/μL of template DNA (2 μL), and 0.5 μL of Phusion® High-Fidelity DNA polymerase (5U/μL) (New England BioLabs, USA) in a final reaction volume of 50 μL. The PCR cycle parameters were as follows: initial denaturation at 98 °C for 1 min followed by 30 cycles at 98 °C for 10 s, annealing at 57 °C for 45 s, extension at 72 °C for 1 min, and final extension at 72 °C for 10 min followed by storage at 4 °C. The PCR products obtained were further purified using QIAquick Gel Extraction Kit (QIAGEN, Germany) and subsequently sequenced. Sites for restriction enzyme *BamHI* and *SalI* were engineered at their forward and reverse ends respectively for direct cloning into pET28. All deletion fragments were ligated into pET-28a (Novagen) *E. coli* expression vector with N- terminal 6 Histidine tag. Ligated plasmid was transformed into chemical competent cells of *E. coli* BL21 (DE3) strain by following the protocol of Sambrook et al. (Russell, 2006).

Table 1
Primers used for amplification of deletion clones.

Name	Primer sequence (5′- 3′)
SphKf	TAGGATCCATGGATCGTCAGCCGGAGAGG
SphKf1	TAGGATCCATGCACCTCGTCTCTCTCGGTCG
SphKf2	TAGGATCCATGGCCGATCGGAATGGTCCCTG
SphKf4	ATGGATCCATGATCCGAGGTCGTACACGTTC
SphKr1	ATGTCGACTTACCGACCGAGAGAGACGAG
SphKr2	ATGTCGACTTAGGGACCATTCCGATCGGC
SphKr4	ATGTCGACTTATTACTTCCTCTTGAAATATC
SphKr5	ATGTCGACTTAATACATCCATGGACCTAACA
SphKr	ATGTCGACTTATTCAGGAGAGAAGAGAGTGGC
Sphkf14.6	TACGATGGTATTGTTTGTCTTGTTGAGGTTGTAAATGG
Sphkr14.6	ATTTACAACCTCAACAAGACAAACAATACCATCGTATT
Sphk1f14.3	TACGATGGTATTGTTTGTGACGGTATCCTTGTTGAGGT
Sphk1r14.3	CTCAACAAGGATACCGTCACAAACAATACCATCGTATT
Sphk1f14.4	TACGATGGTATTGTTTGTGGTATCCTTGTTGAGGTTGT
Sphk1r14.4	AACCTCAACAAGGATACCACAAACAATACCATCGTATT

Note: Underline nucleotides represent the restriction site.

Expression of AtSPHK1 and deletion mutant proteins were induced by 0.4 mM isopropyl-1-thio-D-galactopyranoside (IPTG) at 20 °C for 12 h. 250 ml cell culture was harvested by centrifugation at 6000 × *g* at 4 °C for 10 min and resuspended in 10 ml of lysis buffer containing 10 mM Tris (pH 8.0), 140 mM NaCl and 10 mM Imidazole. Sonication of samples was carried out on ice using six times bursts of 10 s each at 200–300 W with cooling interval of 15 s between each burst. Protein purification was carried out via Ni-NTA-agarose (Qiagen) beads according to manufacturer's protocol. The molecular weights of the proteins were determined using 12% SDS-PAGE gel by using molecular weight standards protein ladder (Bio-Rad). The proteins were stained with Coomassie brilliant blue dye and their concentration was determined by Bradford protein estimation assay (Bradford, 1976). The proteins were transferred from SDS-PAGE to nitrocellulose (NC) membrane for 1 h at 200 mA. The membrane was incubated with 5% fat-free milk in TBST [10 mM Tris (pH 8.0), 140 mM NaCl, and 0.1% (v/v) Tween 20] for 1 h. The membrane was washed thrice with TBST and incubated with anti-his mouse monoclonal antibody (Sigma Aldrich). The membrane was washed again and followed by incubation with goat anti-mouse IgG antibody (Santa Cruz Biotechnology) conjugated to Alkaline phosphatase. Color was developed using alkaline phosphatase conjugate substrate kit (Bio-Rad).

2.2. Protein lipid overlay assay

The Protein lipid overlay assay was performed as described by Zhang et al. (2004) with certain modification (Zhang et al., 2004). 10 μg and 30 μg of Phosphatidic acid (Avanti polar lipids, USA) were spotted onto NC membrane and dried at 24 °C for 1 h. The NC membrane was blocked with 5% fat-free milk in TBST for 1 h. Then, NC membranes were incubated with 30 μg of His_{6x}-tagged AtSPHK1 and other truncated protein in TBST at 4 °C for overnight with gentle shaking. The membranes were washed thrice in TBST and incubated with anti-his mouse monoclonal antibody followed by another round of washing and incubation with goat anti-mouse IgG antibody conjugated to Alkaline phosphatase. Color development was performed using alkaline phosphatase conjugate substrate kit (Bio-Rad) (Dowler et al., 2002).

2.3. Random mutagenesis

Random mutagenesis is a powerful technique used for altering the properties of enzyme such as change in stability, reaction specificity, binding affinity and substrate specificity of enzyme. It is employed to identify critical amino acids essential for enzyme functionality. Random mutagenesis also enables the development of new enzymatic properties without any need of structural details of the targeted enzyme and creates unique mutations (Fujii, 2004). Random mutations in AtSPHK1 were introduced via GeneMorph® II EZClone Domain mutagenesis kit (Agilent technologies). AtSPHK1 cDNA cloned in pET28a following manufacturer's protocol. Random mutagenesis was carried out to introduce only one or two mutation/s per kb length. AtSPHK1 was used as