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The plant defensin NaD1 introduces membrane disorder through a specific interaction with the lipid, phosphatidylinositol 4,5 bisphosphate

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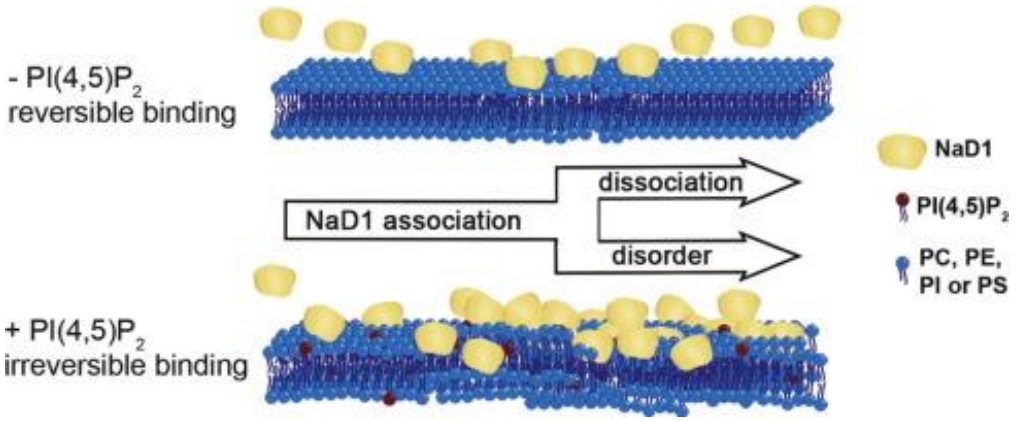
Abstract

- Binding of the plant defensin NaD1 to bilayers containing PI(4,5)P_2 is irreversible.
- Binding of NaD1 to bilayers causes membrane disorder.
- Defensins that bind PI(4,5)P_2 have co-evolved a protective C-terminal propeptide.

Plant defensins interact with phospholipids in bilayers as part of their cytotoxic activity. Solanaceous class II defensins with the loop 5 sequence pattern "S–[KR]–[ILVQ]–[ILVQ]–[KR]–[KR]" interact with PI(4,5)P_2. Here, the prototypical defensin of this class, NaD1, is used to characterise the biophysical interactions between these defensins and phospholipid bilayers. Binding of NaD1 to bilayers containing PI(4,5)P_2 occurs rapidly and the interaction is very strong. Dual polarisation interferometry revealed that NaD1 does not dissociate from bilayers containing PI(4,5)P_2. Binding of NaD1 to bilayers with or without PI(4,5)P_2 induced disorder in the bilayer. However, permeabilisation assays revealed that NaD1 only permeabilised liposomes with PI(4,5)P_2 in the bilayer, suggesting a role for this protein–lipid interaction in the plasma membrane permeabilising activity of this defensin. No defensins in the available databases have the PI(4,5)P_2 binding sequence outside the solanaceous class II defensins, leading to the hypothesis that PI(4,5)P_2 binding co-evolved with the C-terminal propeptide to protect the host cell against the effects of the tight binding of these defensins to their cognate lipid as they travel along the secretory pathway. This data has allowed us to develop a new model to explain how this class of defensins permeabilises plasma membranes to kill target cells.



1. Introduction

Plants, insects and animals produce a large arsenal of cationic peptides as a first line of defence against potential bacterial and fungal pathogens [1]. Many function by disrupting the cell membrane of the target pathogen leading to leakage of cellular contents and cell death [2]. However, the mechanism of membrane disruption varies between different antimicrobial molecules and is not always well understood. A number of mechanisms for membrane disruption have been proposed. These include: coating the surface and destabilising the membrane (carpet model), insertion into the membrane and formation of discrete pores by self-association (toroidal pore model) or association with membrane lipids (barrel-stave pore model) (reviewed in [3]). In 2014, Poon and colleagues described a new mechanism of membrane destabilisation by the plant defensin NaD1 from the ornamental tobacco *Nicotiana* alata. This defensin is a potent antifungal molecule that also targets tumour cells. After exposure to NaD1, tumour cells rapidly form large membrane blebs and the cells burst and die. Lipid strip assays revealed that NaD1 interacts preferentially with the membrane lipid, phosphatidylinositol 4,5 bisphosphate ($PI(4,5)P_2$) and Poon and colleagues [4] went on to solve the crystal structure of the NaD1-$PI(4,5)P_2$ complex. They described a unique arch shaped oligomer composed of seven dimers of NaD1 that cooperatively bound the anionic head groups of 14 $PI(4,5)P_2$ molecules through a unique 'cationic grip' configuration. NaD1:$PI(4,5)P_2$ oligomers also formed readily in solution producing long string-like fibrillar structures that were visualised by transmission electron microscopy. $PI(4,5)P_2$ binding has been described for a number of other protein domains including the pleckstrin homology (PH) domain found in hundreds of proteins [5], the epsin NH_2-terminal homology domain (ENTH) [6] found in proteins that assemble the clathrin lattice during endocytosis, FERM domains that link actin to the plasma membrane [7] and tubby domains of G-protein coupled receptors [8]. However, NaD1 has no sequence or structural homology to any of these aside from having a positive charge in the $PI(4,5)P_2$ binding pocket. These observations led to the hypothesis that NaD1 permeabilises cells by forming a complex with $PI(4,5)P_2$ on the inner leaflet of the plasma membrane that leads to membrane blebs and membrane rupture, possibly by disruption of cytoskeleton–membrane interactions that occur through $PI(4,5)P_2$ [4].

The defensins are one of the largest families of innate immunity peptides produced by plants. They are produced by all plant species that have been examined to date and by most plant tissues [9]. Plant defensins have a conserved three dimensional structure, called a cysteine stabilised alpha-beta motif (CSαβ), which is composed of an alpha-helix and three beta-strands, stabilised by four highly conserved disulphide bonds [9]. Apart from the conserved cysteine residues and a couple of other residues that are essential for structure, the defensins share little sequence homology, especially in the loops that are presented on the surface of the molecule. This variation in sequence explains the diversity of functions that have been described for plant defensins including: antifungal activity, protease and α-amylase inhibitory activities and roles in plant development and pollen tube guidance (reviewed in [10]). Many of the plant defensins are potent antifungal molecules, but the wide diversity in sequence reflects several different mechanisms of action [10]. Plant defensins are grouped into two classes, I and II, based on the absence or presence of a C-terminal propeptide (CTPP) that targets the defensin to the vacuole of the plant cell [9]. Class I defensins lacking the CTPP are transported to the extracellular space where they provide a first line of defence against invading fungal pathogens. Targeting of class II defensins to the vacuole by the CTPP is crucial for preventing phytotoxicity [11]. Here we compare the lipid interaction of two *N. alata* defensins, the class I defensin NaD2 and the class II defensin NaD1.

Plant defensins can also be classified according to their lipid binding specificity: NaD1 and related class II defensins from the Solanaceae, such as TPP3 from tomato, target $PI(4,5)P_2$ [4], [12]. Sphingolipid binding

has been described for another subset of plant defensins, although the exact contribution of the lipid binding to the antifungal activity is still unknown. For most of these defensins the involvement of sphingolipids in the mechanism of action was identified using mutant fungi with disruptions of genes in sphingolipid biosynthesis pathways [13], [14], [15], [16], [17], [18].

The kinetics of the membrane interaction has only been determined for two plant defensins. In the first example, the interaction between the pea defensin PsD1 and membranes containing the sphingolipid glucosylceramide (GluCer) was characterised using surface plasmon resonance (SPR) [19] and NMR spectroscopy [17]. These analyses revealed an increased affinity of PsD1 for membranes containing GluCer, with rapid association and dissociation kinetics and a change in the conformation of the defensin associated with GluCer binding. The second example, MtDef4 from *Medicago truncatula* binds to phosphatidic acid (PA) [20]. SPR experiments revealed rapid association and dissociation of MtDef4 with PA containing membranes similar to those reported for PsD1 and GluCer containing membranes, although binding of MtDef4 to PA was not lipid specific. MtDef4 also bound to phosphatidylinositol phosphates, especially PI(3,5)P_2 on lipid strips. The RGFR(RR) sequence in the surface loop 5 region of MtDef4 is crucial for PA binding. This was demonstrated when PA specificity was lost when loop 5 was replaced with the corresponding loop from MsDef1, a defensin that does not bind PA [20].

The interaction between NaD1 and PI(4,5)P_2 has been demonstrated using lipid strips, liposome pull-down assays and release of ATP from PC:PI(4,5)P_2 liposomes [4], [12]. Here, we have characterised the biophysical interaction between NaD1 and lipids including PI(4,5)P_2 in a more complex lipid bilayer representative of fungal membranes and describe a novel pattern of bilayer disruption not yet reported for an antimicrobial peptide or detergent.

2. Experimental procedures

2.1. Protein sources

The defensins NaD1 and NaD2 were purified from the flowers of *N. alata* as described in Dracatos et al. [21] and Lay et al. [22]. CP29 and Histatin 5 were purchased from GenScript (Hong Kong China). TPP3 and RsAFP2 were expressed as secreted recombinant protein in the methylotropic yeast *Pichia pastoris* and purified using an SP Sepharose column (GE Healthcare Biosciences) as described by Lay et al. [23]. Reduced and alkylated (R&A) NaD1 was produced as described by Poon et al. [4]

2.2. Lipid sources

Lipids were purchased from Avanti Polar Lipids Inc. and included: l-α-phosphatidylcholine (PC, chicken egg), l-α-phosphatidylethanolamine (PE, chicken egg), l-α-phosphatidylinositol (PI, bovine liver), l-α-phosphatidylserine (PS, porcine brain), l-α-phosphatidylinositol-4,5-bisphosphate (PI(4,5)P_2, porcine brain), l-α-phosphatidic acid (PA, chicken egg), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (POPE), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), and 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoserine (POPS). Lipids were dissolved in chloroform to 2 mM except PI(4,5)P_2, which was dissolved in chloroform:methanol:water (20:9:1) to a 2 mM stock solution.

2.3. Protein–lipid overlay assay

Binding of NaD1 and NaD2 to lipids immobilised on strips was performed using PIPstrips™ (Echelon) and 1.5 μg/mL of defensin in the lipid overlay. Defensin binding was detected using NaD1 [26] or NaD2 [21] antibodies as described previously in [4].

2.4. Liposome preparation

Unilamellar liposomes were prepared as outlined by Zhang et al. [24]. Lipids were combined to achieve the desired molar ratio of lipid components as outlined in Table 1. PC:PG based liposomes were used to represent simple membranes, PC:PE:PS:PI based liposomes were used to represent fungal membranes [25]. The lipid mixtures were rehydrated to 14 mg/mL in HEPES buffer (50 mM, pH 7) for 2 h at RT with occasional vortexing, before the mixtures were sonicated using a Vibra Cell™ sonicator (Sonics and Materials, USA) for 5 min until the mixture cleared.

Table 1. Composition of liposomes. The ratio of lipids used for preparing small unilamellar liposomes.

Lipid composition	Ratio
PC:PE:PS:PI	52:30:10:8
PC:PE:PS:PI:PI(4,5)P_2	51:30:10:8:1
	50:30:10:8:2
	48:30:10:8:4
PC:PE:PS:PI:PA	50:30:10:8:2
	48:30:10:8:4
PC:PG	75:25
PC:PG:PI(4,5)P_2	77.5:20:2.5
	75:20:5
PC:PG:PA	77.5:20:2.5
	75:20:5

2.5. Liposome binding assay

Liposomes were washed twice in HEPES buffer before use. The liposomes at 14 mg/mL (50 µL) were incubated with 2 µg of the test protein (2 µL of 1 mg/mL, dissolved in water) for 1 h at 25 °C. The mixture was centrifuged at 16,000 × g for 5 min and 30 µL of the supernatant was reserved for analysis. The liposome pellet was washed twice with 200 µL of buffer before it was resuspended in 50 µL of HEPES buffer, then analysed by SDS-PAGE and immunoblotting using the appropriate antibody.

2.6. Dynamic light scattering of liposomes

A 400 µL sample of liposomes (1.4 mg/mL) was incubated with test protein (0–8 µM) for 30 min in a semimicro cuvette (Greiner Bio-one). The liposome samples were characterised on a Malvern Zetasizer Nano ZS dynamic light scattering instrument using He–Ne laser 633 nm and Zetasizer software, version 7.01 (Malvern Laboratories Ltd, Malvern, UK). Four measurements were conducted for each sample and the data was analysed using GraphPad Prism® 6.

2.7. Calcein release from liposomes

Disruption of liposomes by antifungal proteins was observed using calcein-loaded liposomes as described by Zhang et al. [24] and van der Weerden et al. [25] except that fluorescence measurements were taken every 30 s for 390 s. Change in fluorescence was monitored over time after the addition of Triton X-100 (final concentration 0.1%) or test peptide (final concentration 10 µM).

2.8. Dual polarisation interferometry (DPI)

Production of liposomes for DPI was performed as described by Lee et al. [26]. Lipid mixtures of POPC:POPS:POPE:PI and POPC:POPS:POPE:PI(4,5)P_2 in a ratio of 56:10:30:4 were prepared and rehydrated in 10 mM MOPS, pH 7 with 150 mM NaCl at 40 °C. Dual polarisation interferometry (DPI) was conducted as described by Lee et al. [26]. Once a stable bilayer was obtained on the chip surface at 20 °C, NaD1 (160 µL of 16, 8, 4, 2 or 1 µM) was injected at 40 µL/min for 4 min followed by a dissociation for 30 min in 10 mM MOPS buffer pH 7 with 150 mM NaCl. The chip surface was regenerated and cleaned as described in Lee et al. [26] and a new bilayer was deposited for each NaD1 injection.

2.9. Calculation of optical birefringence and mass of an anisotropic layer

Birefringence was obtained from DPI by calculating the difference between the two effective refractive indices, namely the refractive index (RI) of the transverse magnetic (TM) waveguide mode (nTM) and the RI of the transverse electric (TE) waveguide mode (nTE). The birefringence values represent an averaged measurement of the degree of alignment and packing order of lipid molecules and are highly sensitive to small changes in these parameters. The effective birefringence (nTM–nTE) was determined by fixing the RI of the deposited layer to 1.47 indicative of uniform layer coverage as determined by the absence of bovine serum albumin binding at 1 mg/mL in running buffer [26]. The De Feijter formula [27] was used to calculate the mass of adsorbed lipid and peptide layers using 0.135 mL/g and 0.182 mL/g for the specific refractive index increment (dn/dc) of lipid and peptide, respectively. The refractive index of the MOPS buffer (nbuffer) was 1.3349 (T = 20 °C). An average bilayer thickness of 46.1 ± 0.4 Å and a birefringence of 0.0198 ± 0.0005 were obtained for POPC:POPS:POPE:PI (n = 10) while an average bilayer thickness of 48.6 ± 0.5 Å and a birefringence of 0.0191 ± 0.0003 were obtained for the POPC:POPS:POPE:PI(4,5)P_2 membrane (n = 10).

2.10. Alignment of defensin sequences

Defensin sequences were gathered from the GenBank non-redundant protein sequence database based on matching the sequence regular expression ".{2}C.{5,12}C.{4,8}C.{3}C.{9,11}C.{4,11}C.C.{2,3}C". The sequences were sub-setted based on the presence or absence of the sequence regular expression S[KR][ILVQ]{2}[KR]{2}. The sequences not containing the regular expression were cross-referenced with literature on antifungal activity (abstract contained words matching any of the following regular expressions: fung.* | myco.* | IC50 | anti.* | .*cidal | inhibit.* | survival | charac.* | assay). Those reporting IC50 < 10 µM were used as representative comparison sequences. The sequences were aligned using CysBar [28], which also calculated net charge for the mature and C-terminal prodomain (if present). See Supplementary Table 1 for species names and GenBank identifiers.

3. Results

The lipid binding profile of NaD1 and NaD2 was initially evaluated using immobilised lipids on a 'lipid strip' (Fig. 1A). Consistent with previously published data [4], NaD1 bound preferentially to PI(4,5)P_2 and less well to phosphatidylinositol monophosphates and other diphosphates. NaD2 bound to phosphatidic acid (PA) and the phosphatidyl inositol monophosphates.

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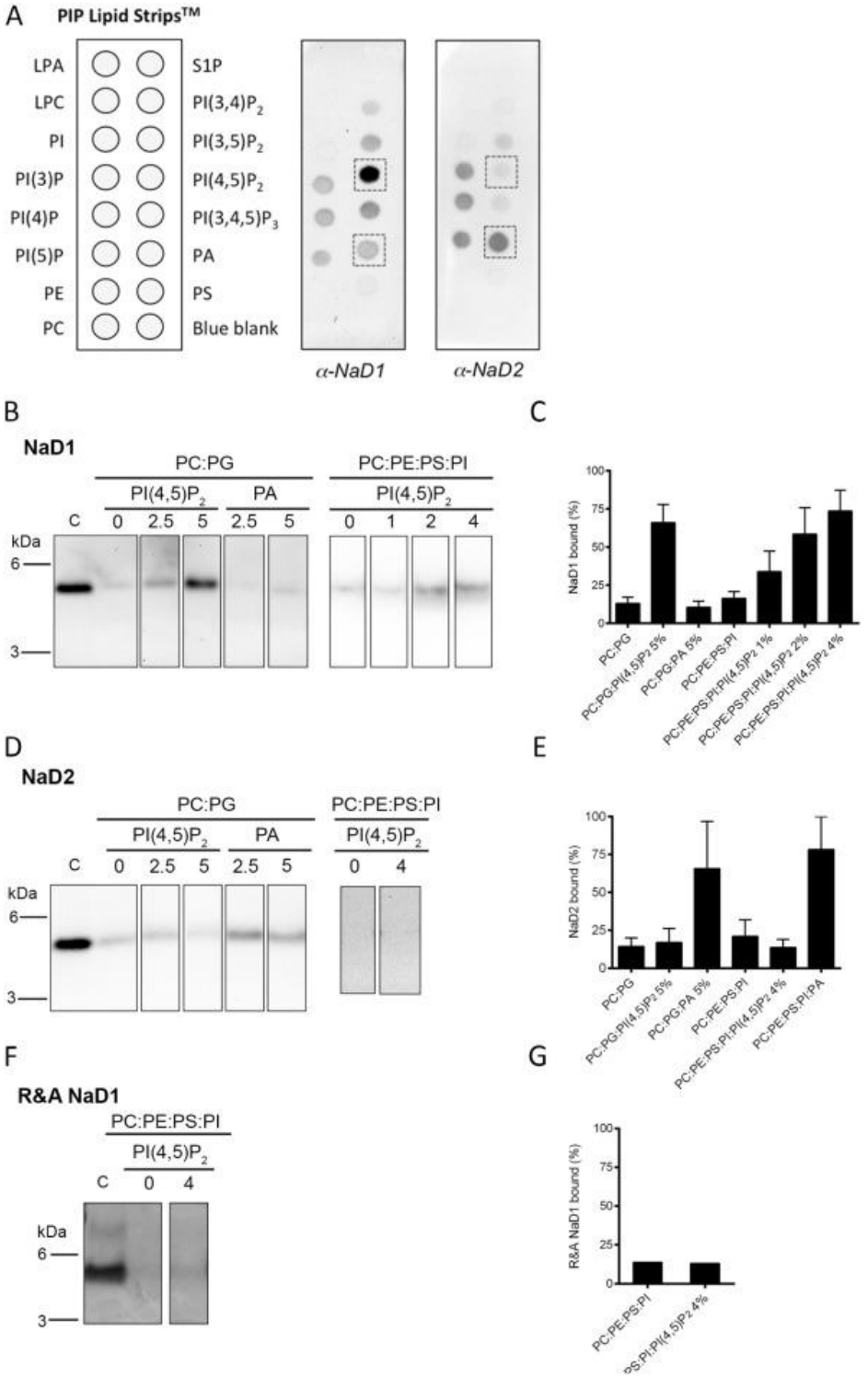

Fig. 1. Binding of NaD1, NaD2 or R&A NaD1 to liposomes containing PI(4,5)P₂ or PA. (A) Binding of NaD1 and NaD2 to PIPstrips™ was detected by Western blot analysis with α-NaD1 IgG, or α-NaD2 IgG. Boxed are the lipid spots of PA and PI(4,5)P₂. Lysophosphatidic acid (LPA), lysophosphocholine (LPC), phosphatidylinositol (PI), phosphatidylinositol 3-phosphate (PI(3)P), phosphatidylinositol 4-phosphate (PI(4)P), phosphatidylinositol 5-phosphate (PI(5)P), phosphatidylethanolamine (PE), phosphatidylcholine (PC), sphingosine 1-phosphate (S1P), phosphatidylinositol 3,4-bisphosphate (PI(3,4)P₂), phosphatidylinositol 3,5-bisphosphate (PI(3,5)P₂), phosphatidylinositol 4,5-bisphosphate (PI(4,5)P₂), phosphatidylinositol 3,4,5-triphosphate PI(3,4,5)P₃, phosphatidic acid (PA), phosphatidylserine (PS) and

lipid negative control (blue blank). (B) Binding of NaD1 to liposomes composed of PC:PG or PC:PE:PS:PI containing various amounts of $PI(4,5)P_2$ or PA. Western blot analysis of liposome pellet with α-NaD1 antibody revealed that NaD1 bound to liposomes when $PI(4,5)P_2$ was present. (C) Densitometry analysis of bands from panel B. The amount of NaD1 that bound increased with $PI(4,5)P_2$ concentration. (D) Binding of NaD2 to liposomes. NaD2 bound to PC:PG liposomes when PA was present at 2.5 or 5% but did not bind to PC:PG or PC:PC:PE:PI liposomes when $PI(4,5)P_2$ was present. Bound NaD2 was detected using the α-NaD2 antibody and Western analysis. (E) Densitometry analysis of bands from panel D. (F) Binding of reduced and alkylated (R&A) NaD1 to liposomes. No binding of R&A NaD1 was detected even in the presence of $PI(4,5)P_2$. R&A NaD1 was detected by Coomassie Blue staining. (G) Densitometry analysis of bands from panel F. C is the defensin only control. The numbers above the lanes indicate the percentage of $PI(4,5)P_2$ or PA in the liposomes. Images are representative of three separate experiments. Error bars on densitometry analysis graphs are SEM.

To establish whether this binding specificity also occurred in lipid bilayers, the defensins were added to liposomes with or without PA or $PI(4,5)P_2$, and binding was inferred if the protein segregated with the pelleted liposomes. NaD1 bound to liposomes that contained $PI(4,5)P_2$ in a concentration dependent manner. An increase in the concentration of $PI(4,5)P_2$ from 1% to 4% in PC:PE:PS:PI liposomes, the lipid ratio set to mimic fungal membranes, led to an increase in the amount of NaD1 that bound. Binding of reduced and alkylated NaD1 to $PI(4,5)P_2$ containing liposomes was assessed to determine whether the interaction was dependent on the disulphide bond stabilised three dimensional structure of the defensin. No reduced and alkylated NaD1 was detected in the pellet fraction. NaD2 bound weakly to PA liposomes and not at all to $PI(4,5)P_2$ containing liposomes (Fig. 1).

The ability of NaD1 and NaD2 to disrupt liposomes of varied lipid compositions was monitored using the calcein release assay. Addition of Triton X-100 to the calcein-loaded liposomes resulted in a sharp increase in fluorescence that peaked within the first 30 s for all liposomes tested (Fig. 2). The percentage of calcein released from the liposomes of various lipid compositions was calculated for each of the antifungal proteins tested relative to release by Triton X-100 (Fig. 2). The melittin–cecropin hybrid, CP29 (10 μM) [29], induced more than 50% calcein release from all the liposomes irrespective of lipid composition. In contrast, addition of 10 μM NaD2 resulted in less than 5% release of calcein across all of the liposomes including those containing its cognate lipid, PA. NaD1 released less than 10% of the calcein from PC:PG and PC:PE:PS:PI liposomes without $PI(4,5)P_2$ (Fig. 2). However, more than 50% of the calcein was released after the addition of NaD1 (10 μM) to PC:PE:PS:PI liposomes with 4% $PI(4,5)P_2$ (Fig. 2B). This was comparable to the level of permeabilisation obtained with CP29. Permeabilisation of liposomes by NaD1 was dependent on the $PI(4,5)P_2$ content as only 10 % calcein release was observed when $PI(4,5)P_2$ levels were lowered to 1%.

The liposome permeabilisation experiments were extended from the two plant defensins to a variety of other antifungal proteins (Fig. 2). Less than 5% liposome permeabilisation was obtained with reduced and alkylated NaD1 (R&A NaD1), NaD2, the plant defensin RsAFP2, and the human antifungal protein Histatin 5 in the presence or absence of $PI(4,5)P_2$. The defensins, TPP3 and NaD1 both bound preferentially to $PI(4,5)P_2$ on lipid strips and displayed enhanced liposome permeabilisation when $PI(4,5)P_2$ was present in the liposome (Fig. 2) which is consistent with previous observations [4], [12].

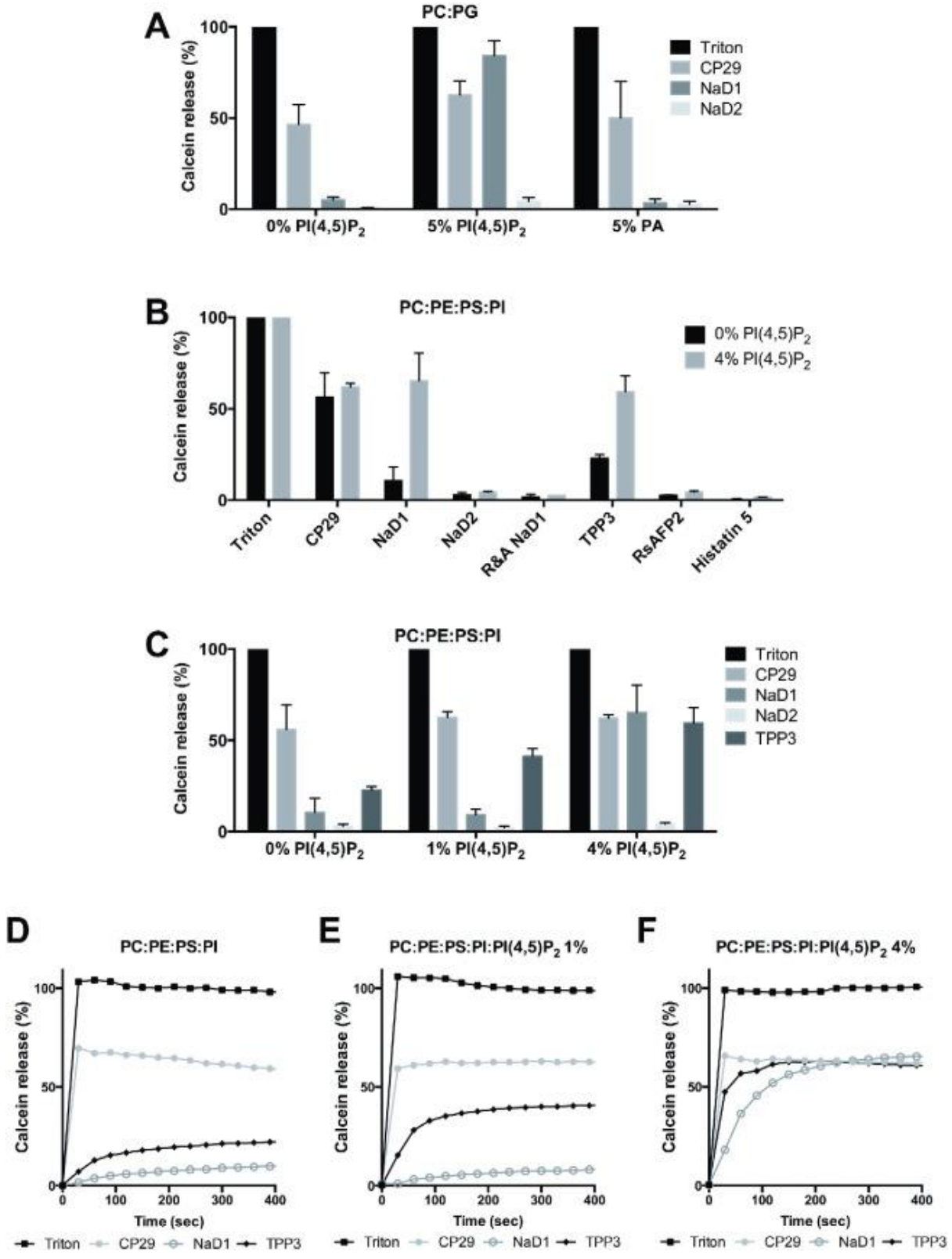

Fig. 2. Permeabilisation of liposomes containing PI(4,5)P2 or PA by antifungal peptides. Calcein release was measured by the increase in fluorescence after treatment of calcein-entrapped liposomes with Triton X-100 or antifungal peptides. (A) Calcein release from PC:PG liposomes with or without PA or PI(4,5)P2 making up 5% of the total lipid composition of the liposomes. When PI(4,5)P2 was present NaD1 permeabilised liposomes as effectively as CP29. NaD2 did not release calcein from liposomes containing

PA. (B) The release of calcein from calcein-loaded PC:PE:SI:PI liposomes with or without PI(4,5)P2 by Triton X-100, CP29 and the plant defensins NaD1, NaD2, reduced and alkylated NaD1 (R&A NaD1), TPP3 and RsAFP2, and the human antifungal protein Histatin 5. (C) Permeabilisation by NaD1 and TPP3 increased with PI(4,5)P2 concentration in the liposome. (D, E, F) Rate of permeabilisation of PC:PE:PS:PI liposomes with increasing amounts of PI(4,5)P2. The rate of permeabilisation by TPP3 and NaD1 increased with PI(4,5)P2 concentration, with TPP3 producing more rapid release of calcein than NaD1. Calcein release by 0.1% Triton X-100 was set as 100% calcein release and calcein release from all the proteins was plotted relative to this. Error bars representing SEM, n = 2.

The effect of the *N. alata* defensins on the size of liposomes was examined by dynamic light scattering (DLS) (Fig. 3). Triton X-100 caused a large decrease in the size of liposomes as expected for a detergent, average liposome size decreased from approximately 90 nm to 20 nm in diameter. When NaD1, NaD2 or TPP3 was added to PC:PE:PS:PI liposomes a small increase in size was observed. However, addition of NaD1 or TPP3 led to a significant increase in size when 4% PI(4,5)P_2 was present in the liposome. NaD1 increased average liposome diameter from 90 nm to 136 nm and TPP3 caused an increase to 121 nm. In contrast, the addition of NaD2 to liposomes with PI(4,5)P_2 did not cause a change in size. Surprisingly a difference in liposome size was also observed upon treatment with CP29 but only with the liposomes containing PI(4,5)P_2. This change in size was much smaller than that observed for NaD1 or TPP3.

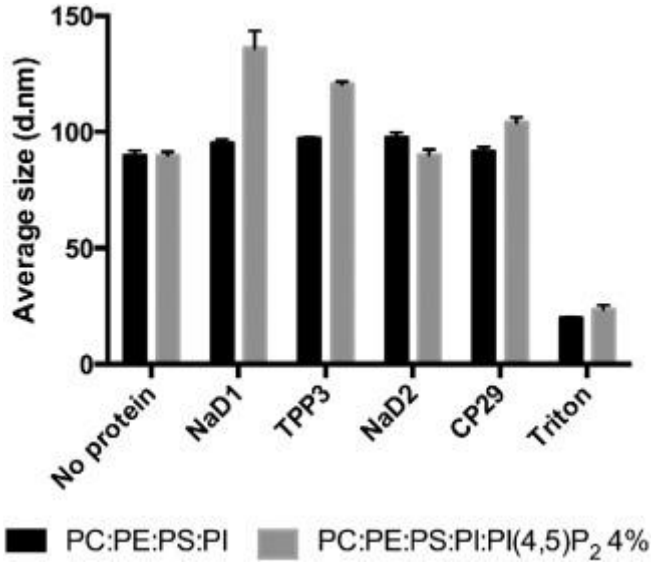

Fig. 3. The effect of defensins on the size distribution of liposomes. The size of the PC:PE:PS:PI liposomes in the presence of Triton X-100, the plant defensins NaD1, TPP3 or NaD2 and the antifungal peptide CP29 was determined by dynamic light scattering (DLS). Addition of the plant defensins resulted in a slight increase in the size of the PC:PE:PS:PI liposomes. However, when PI(4,5)P2 was present there was a larger increase in liposome size after incubation with NaD1 or TPP3. Graphs are representative of 3 separate experiments with fresh liposomes. Error bars are SD from one experiment where the sample was run 4 separate times.

The technique of dual polarisation interferometry (DPI) was used to gain a better understanding of the role of PI(4,5)P_2 in the interaction of NaD1 with lipid bilayers. DPI provides a simultaneous measure of the amount of NaD1 bound to the lipid bilayer and the impact of NaD1 binding on the molecular order of the bilayer. NaD1 associated rapidly with the bilayer containing PI(4,5)P_2 and with greater affinity than to the PI containing bilayer (Fig. 4). The amount of NaD1 that bound to the PI(4,5)P_2 bilayer increased with increasing concentrations of NaD1. At 2 µM, NaD1 binding increased progressively over the entire 4 min period of the protein injection, and once bound NaD1 did not dissociate from the PI(4,5)P_2 bilayer. At

concentrations greater than 4 µM, NaD1 associated quickly with the PI(4,5)P_2 bilayer, that is, within the first minute of protein injection. This rapid association was followed by a very slow association for the remaining NaD1 injection. Twice the amount of NaD1 (2 ng/mm^2) bound to the bilayer with PI(4,5)P_2 and it did not dissociate after 10 min of washing. In contrast, in the absence of PI(4,5)P_2, NaD1 that bound to the PI containing bilayer dissociated faster and about 70% had dissociated from the bilayer after 10 min of washing. The rate of association of NaD1 with the bilayer containing PI(4,5)P_2 increased as the concentration of NaD1 was raised from 1 to 16 µM. The total amount of NaD1 that bound plateaued at about 2 ng/mm^2 at NaD1 concentrations of 4 µM and above (Fig. 4).

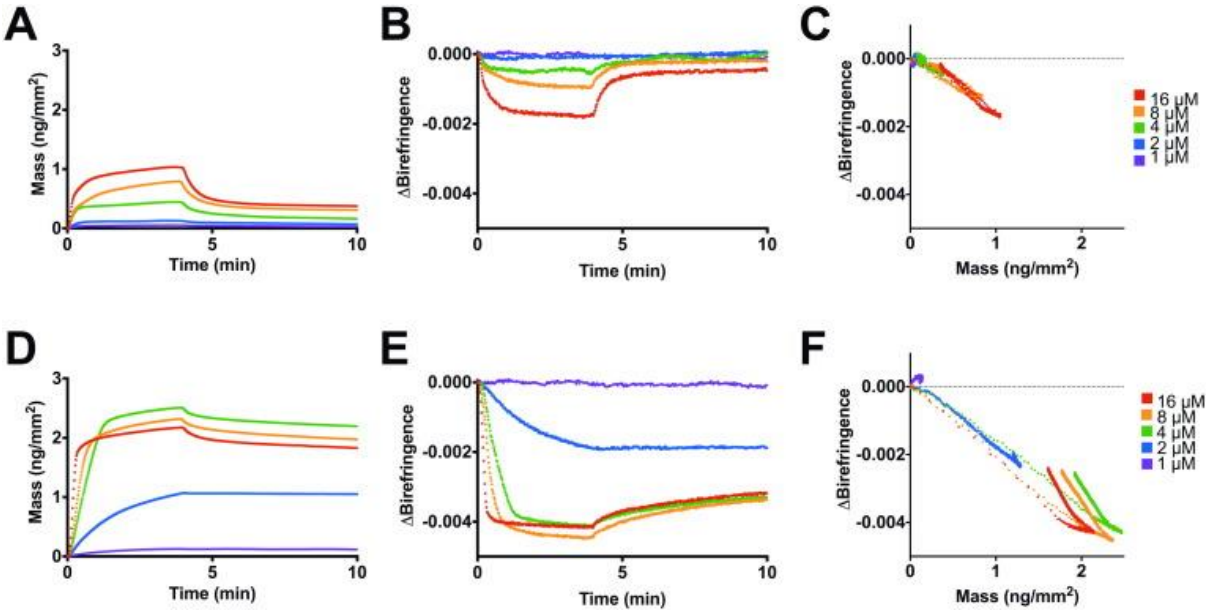

Fig. 4. Binding kinetics of NaD1 to lipid bilayers. Dual polarisation interferometry was used to investigate the effect of NaD1 on lipid bilayers. Kinetics of binding and dissociation of NaD1 to a supported lipid bilayer composed of POPC:POPS:POPE (56:10:30:4) with either (A) PI or (D) PI(4,5)P2. NaD1 (1–16 µM) was injected for 4 min, followed by washing to monitor dissociation of NaD1 from the bilayer. Association and maximum amount of defensin bound were greater for the bilayer containing PI(4,5)P2. A significant proportion of NaD1 dissociated from bilayers without PI(4,5)P2 whereas only minimal dissociation was observed for bilayers that did contain PI(4,5)P2. Real-time changes in the birefringence of the bilayer composed of POPC:POPS:POPE with either (B) PI or (E) PI(4,5)P2 that were induced by the addition of NaD1 were also monitored. NaD1 was injected for 4 min, followed by washing to monitor dissociation of NaD1 from the bilayer. A decrease in bilayer order occurred (decrease in birefringence) after the addition of NaD1 and was more pronounced with the bilayer containing PI(4,5)P2. The bilayer order was restored overtime in the PI bilayer but not in the PI(4,5)P2 containing bilayer. Birefringence with respect to amount of NaD1 bound was also plotted. In bilayers composed of POPC:POPS:POPE with either (C) PI or (F) PI(4,5)P2 bilayer birefringence decreased as more NaD1 bound. Dissociation of NaD1 from the bilayers is indicated by a sharp turn in the graph. Addition of high concentrations of NaD1 (4, 8 or 16 µM) quickly reduced bilayer order (far spacing between points) when PI(4,5)P2 was present.

The real-time changes in birefringence (membrane order) that occurred after the addition of NaD1 to POPC:POPS:POPE bilayers containing either PI or PI(4,5)P_2 are displayed in Fig. 4B and E. There was a linear relationship between the amount of NaD1 bound and rate and extent of the decrease in membrane order to both the PI and PI(4,5)P_2 containing bilayers, that was more evident with the latter. As NaD1 dissociated from the PI-containing bilayer, the order of the bilayer was almost restored to the initial condition. In contrast, at concentrations of 2 µM and above, NaD1 did not fully dissociate from the PI(4,5)P_2 bilayers and the birefringence of the bilayers did not return to the starting condition, consistent with permanent loss of

bilayer order (Fig. 4C and F). A diagram of this interaction between NaD1 and the lipid bilayers determined by DPI is displayed in Fig. 5.

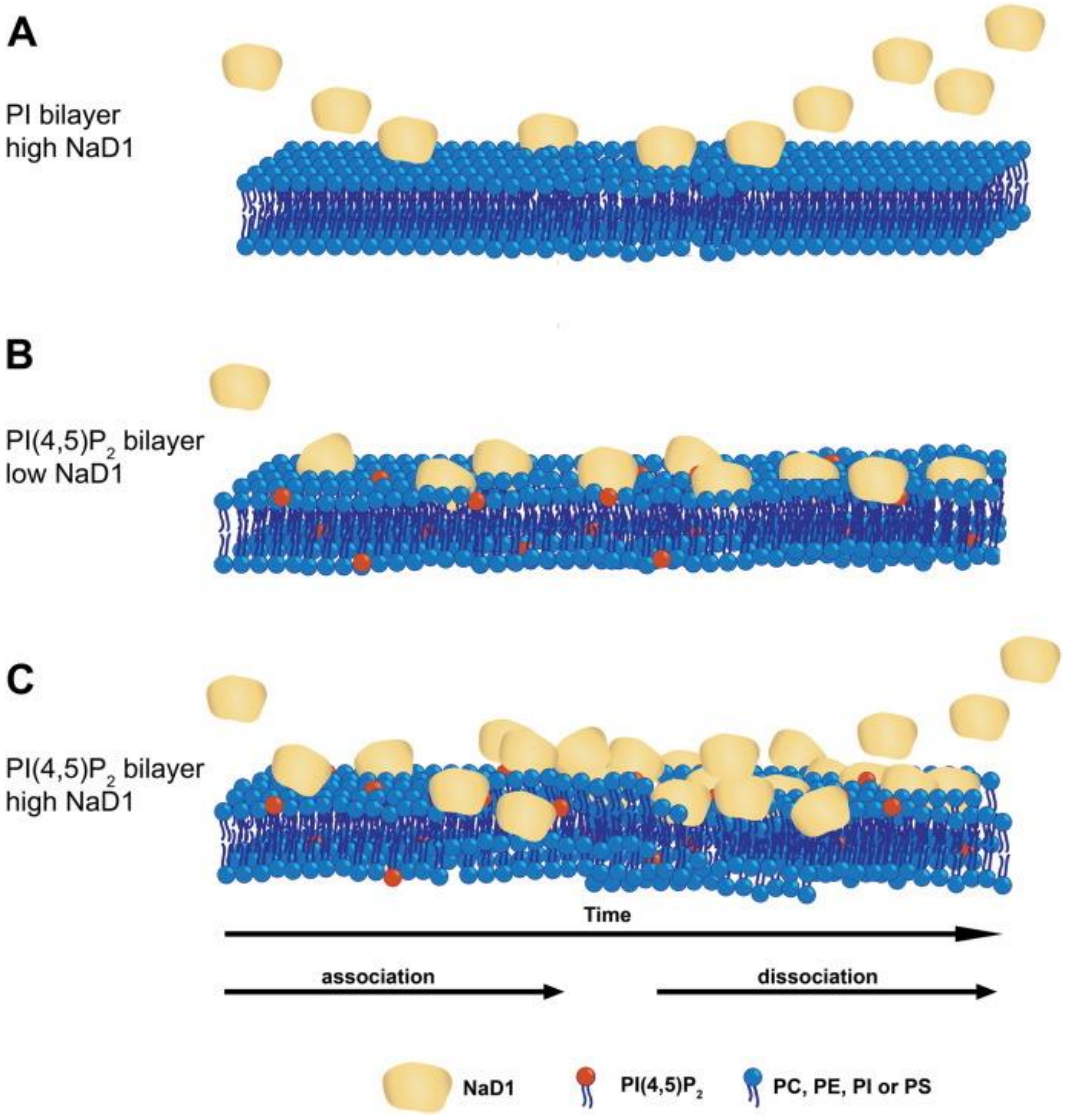

Fig. 5. Interaction of NaD1 with PI and PI(4,5)P2 bilayers. Representation of the interaction of NaD1 with POPC:POPE:POPS:PI or PI(4,5)P2 bilayers as determined by DPI. (A) Association of NaD1 with PI bilayers causes bilayer disorder as more NaD1 binds. After washing, NaD1 dissociates completely from the bilayer and the order of the bilayer returns. (B) At low NaD1 concentrations, NaD1 associates with the PI(4,5)P2 bilayers and causes bilayer disorder similar to the disorder that occurs at high NaD1 concentrations with PI bilayers. However, NaD1 does not dissociate from the bilayer and the disorder of the bilayer remains. (C) At high concentrations of NaD1 with PI(4,5)P2 containing bilayers, NaD1 binds quickly to the bilayer and disrupts the order of the bilayer to twice the extent observed in A or B. NaD1 dissociates slowly from PI(4,5)P2 bilayers, with most of the NaD1 remaining bound and the order of the bilayer not returning.

4. Discussion

Lipid binding and membrane permeabilisation are key components of the mechanisms of action of many defensins. However, unlike many other antimicrobial peptides (AMPs) plant defensins do not disrupt membranes indiscriminately. Here we have focused on the strong interaction between a solanaceous class II defensin, NaD1, and the lipid $PI(4,5)P_2$.

The lipid binding specificity of the class II defensin NaD1 and NaD2, a class I defensin, was initially examined using lipid strips. NaD1 bound to PI(4,5)P_2 as reported previously [4], [12] and less well to the phosphatidylinositol monophosphates PI(3,5)P_2 and PI(3,4,5)P_3. NaD2 bound to PA but this binding was not specific, as a similar amount of NaD2 bound to phosphatidylinositol monophosphates. Although lipid strips are useful for initial assessment of potential lipid binding specificity of proteins, the translation of these results to in vivo systems is questionable because the high concentration and orientation of the lipids on the strip are not representative of biological membranes [30]. Data from lipid strips can be misleading if there are non-specific interactions with exposed hydrophobic acyl chains that are not encountered in a cell. To better understand the interaction of NaD1 and NaD2 with lipids in the context of a biological membrane, lipid vesicles with known lipid ratios set to mimic those of fungal plasma membranes were employed. NaD2 bound very weakly to liposomes containing both PA and PI(4,5)P_2 compared to the strong binding between NaD1 and PI(4,5)P_2 liposomes. Thus, the interaction of NaD2 with PA on lipid strips was not reflected in a preference for PA binding in bilayers. The failure of NaD2 to bind PI(4,5)P_2 in bilayers also led to its use as a negative control in subsequent experiments with NaD1.

Permeabilisation of biological membranes is a key component of the mechanism of action of NaD1 against both fungal and tumor cells [4], [12], [25]. To determine the influence of PI(4,5)P_2 levels on the permeabilisation of membranes by NaD1, calcein release assays were conducted using liposomes of defined lipid composition. NaD1 only permeabilised liposomes that contained PI(4,5)P_2 and the amount of permeabilisation directly related to the level of PI(4,5)P_2 in the liposome, consistent with data published in [12]. In contrast, CP29, an amphipathic antimicrobial peptide (AMP) [29] that acts by forming ion permeable channels in the plasma membrane without the requirement for specific lipid binding, a mechanism described for many AMPs [1], permeabilised all membranes regardless of lipid composition. This was an indication that the mechanism of membrane disruption by NaD1 does not fall into one of the classic mechanisms described for AMPs. NaD1 permeabilised PI(4,5)P_2 containing liposomes at a rate similar to CP29 demonstrating that NaD1 is a potent membrane permeabilising peptide in the presence of cognate lipid. However, not all plant defensins permeabilise liposomes containing their cognate lipid as NaD2 did not release calcein from liposomes containing PA.

Dual polarisation interferometry (DPI) was used to investigate the kinetics of the interaction between NaD1 and phospholipid bilayers as well as the effect of NaD1 binding on the order of the bilayer. NaD1 exhibited moderate and reversible binding in the absence of PI(4,5)P_2. However, both the rate and maximum amount of NaD1 bound increased when PI(4,5)P_2 was present in the bilayer and more significantly, NaD1 failed to dissociate from bilayers containing PI(4,5)P_2, consistent with a very strong interaction between NaD1 and PI(4,5)P_2-containing membranes. These differences in apparent affinity of NaD1 for bilayers with and without PI(4,5)$_{P2}$ demonstrate that initial interactions between NaD1 and phospholipids in a bilayer (presumably through electrostatic interactions) facilitate the subsequent specific interaction between NaD1 and PI(4,5)P_2 molecules in the membrane. This pattern of membrane interaction has been reported for neomycin [31], where electrostatic interactions facilitate initial interactions between neomycin and the outer leaflet of plasma membranes which lack PI(4,5)P_2. Neomycin then translocates the plasma membrane, interacts with PI(4,5)P_2 on the inner leaflet and causes membrane disruption. The low level of dissociation observed with NaD1 and membranes containing PI(4,5)P_2 does not occur with other plant defensins and their cognate lipids. Surface plasmon resonance analysis of the interactions of the defensins PsD1 and MtDef4 with bilayers containing their respective lipid binding partners revealed a rapid association phase similar to that for NaD1 and PI(4,5)P_2 [19], [20]. However, both PsD1 and MtDef4 dissociated from the bilayers following when the wash step was initiated as occurred with NaD1 and bilayers lacking PI(4,5)P_2.

The high affinity of NaD1 for PI(4,5)P_2-containing membranes coincided with a substantial and largely irreversible drop in the order of the bilayer after the addition of NaD1 as measured by birefringence. This contrasted with the bilayer lacking PI(4,5)P_2 where NaD1 dissociated and the membrane order returned to the initial state. These observations support a model where binding of NaD1 to PI(4,5)P_2 in a bilayer induces disorder in the membrane, possibly as a result of NaD1–PI(4,5)P_2 complexes forming oligomers and placing mechanical stress on the lipid bilayer. This pattern of sustained membrane disorder is distinct from the loss of lipid mass observed when the amphipathic α-helical AMP Aurein 1.2 was analysed in a similar system [32]. Again, this supports a novel mechanism of membrane interaction and disruption for NaD1 and related PI(4,5)P_2 binding defensins among AMPs.

Considering NaD1 caused sustained disorder in lipid bilayers containing PI(4,5)P_2, and it forms oligomers with PI(4,5)P_2 [4], DLS was used to determine whether NaD1 had any effect on the size of liposomes. Membrane disruption via a detergent-like mechanism was expected to decrease the size of liposomes as occurred with Triton X-100. None of the proteins tested decreased liposome size and thus were not acting by a detergent like mechanism. Addition of the PI(4,5)P_2 binding defensins, NaD1 or TPP3, caused a small increase in liposome size when PI(4,5)P_2 was present. It is unclear whether this was due to fusion or aggregation of the liposomes.

Reduced and alkylated NaD1 did not interact with PI(4,5)P_2 containing liposomes confirming that the folded structure of this defensin is important for lipid binding. This was expected, as the amino acid side chains of NaD1 that contact the lipid molecule in the crystal structure are not contiguous in the primary sequence and must be brought together by the three dimensional fold (Fig. 6) [4]. They include K4, H33 and four residues, K36, I37, L38 and R40, located on loop 5, the primary lipid binding domain [4]. TPP3, which binds PI(4,5)P_2 through a similar loop 5 sequence to NaD1 (SKLQRK), also permeabilised liposomes containing PI(4,5)P_2 in a lipid concentration dependent manner and is likely to act through a similar mechanism as NaD1 [12]. This is not a general feature of plant defensins, as NaD2 and RsAFP2, which lack the SKILRR loop 5 sequence (Fig. 6) did not permeabilise liposomes containing PI(4,5)P_2. Similarly the human antifungal protein Histatin 5, which also lacks the SKILRR loop sequence, failed to permeabilise PI(4,5)P_2 containing liposomes. Greater permeabilisation of the liposomes with 1% PI(4,5)P_2 by TPP3 compared to NaD1 can be attributed to the greater specificity and possibly affinity of TPP3 for PI(4,5)P_2 (Fig. 2D) that was proposed in [12].

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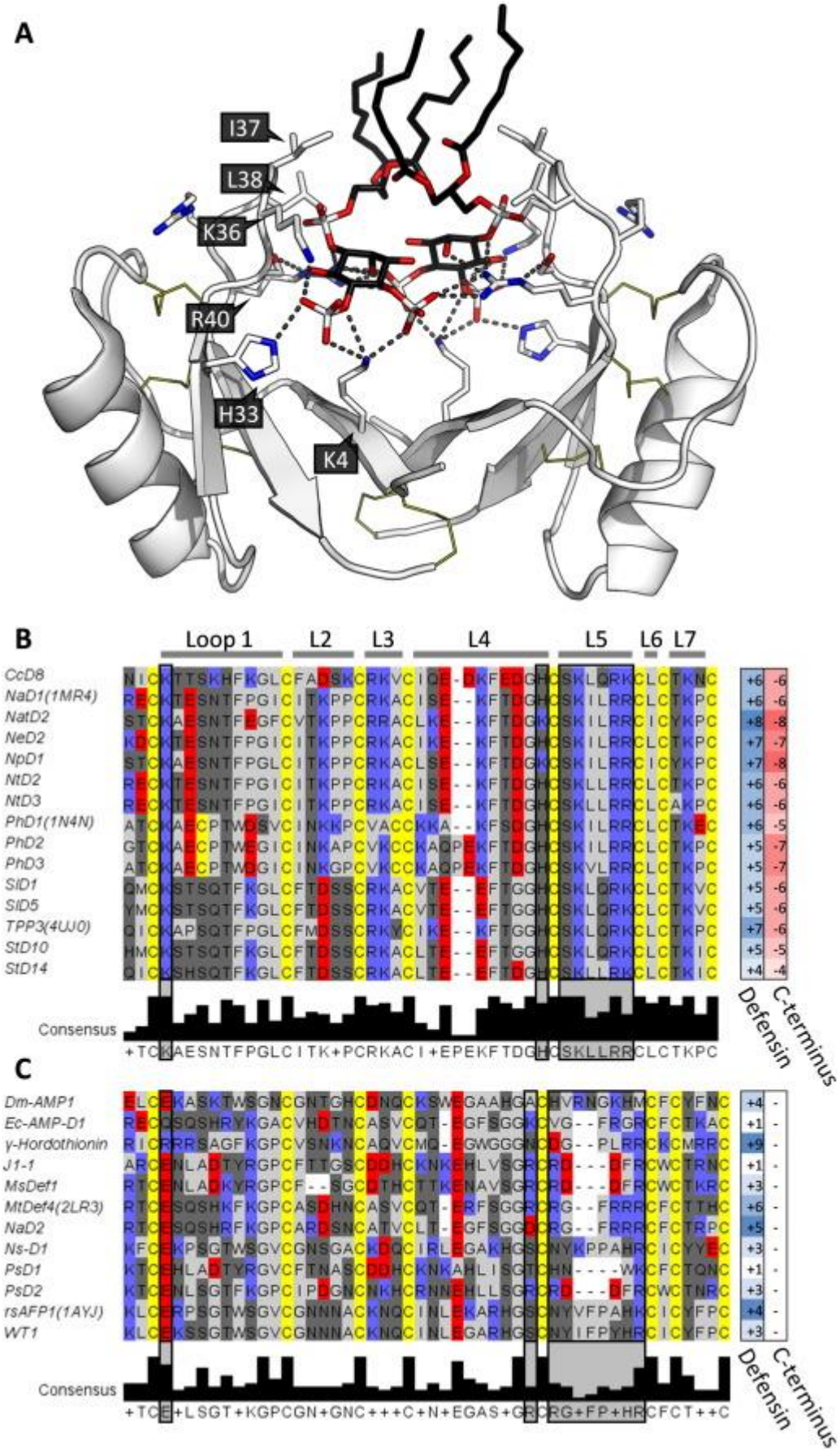

Fig. 6. Conservation of the loop 5 PI(4,5)P2 binding sequence and charge of the C-terminal propeptide in solanaceous class II defensins. (A) Structure of NaD1 complexed with PI(4,5)P2. Polar interactions between protein and lipid are indicated by dashed lines. Side chains involved in these interactions as described in [4] are shown as sticks with nitrogen and oxygen atoms coloured blue and red respectively (PDB: 4CQK). (B) Alignment of full-length defensin sequences containing the loop 5 sequence pattern "S–[KR]–[ILVQ]–[ILVQ]–[KR]–[KR]". Cysteines are coloured yellow. Positive, negative, polar and hydrophobic residues are coloured blue, red, dark grey and light grey respectively. The net charges of the defensin domain and the C-terminal pro-domain are indicated. Loops are the regions between cysteine residues (L1–L7) and are indicated above the alignment. (C) Alignment of defensin sequences with known antifungal activity, but not containing the loop 5 sequence pattern "S–[KR]–[ILVQ]–[ILVQ]–[KR]–[KR]". None have C-terminal pro-domains.

A search of the available sequence data identified 13 additional defensins to NaD1 and TPP3 that possess a sequence similar to SKILRR in loop 5. These defensins also have a conserved lysine residue at position 4 and histidine at position 33 (Fig. 6). These residues contribute to the cationic grip identified in the lipid bound structures of both NaD1 and TPP3 [4], [12] suggesting that there is strong selective pressure for the cationic grip, a motif that has been linked to potent antifungal activity. All of the defensins that possess this cationic grip motif are class II defensins [11], which are produced as precursors with a negatively charged CTPP that targets them to the vacuole for storage. Analysis of the sequences of a selection of class I defensins revealed that although there was some prevalence of positively charged amino acid residues in the region corresponding to the SKILRR loop, there is no conservation of the other cationic grip residues (Fig. 6C). Thus, they are not likely to bind to PI(4,5)P_2 although this has never been tested. Indeed, DmAMP1 binds to the sphingolipid mannosyldiinositolphosphorylceramide [33], MsDef1 and RsAFP2 bind to glucosylceramide [18] [34], and MtDef4 and NaD2 bind to phosphatidic acid [20]. None of these defensins are class II defensins, meaning they lack the CTPP. A likely explanation for this strict conservation of sequence motifs is that the strength of PI(4,5)P_2 binding conveyed by the cationic grip and subsequent membrane lytic activity makes class II defensins phytotoxic, if directed to the extracellular space, and thus, they must be sequestered in the vacuole until required for defence against infection. The negative charge on the CTPP probably functions to counteract the positive charges in the cationic grip of the mature defensin domain to prevent unwanted interactions with lipids during transport through the secretory pathway and into the vacuole [11]. The overall positive charge of class I defensins often exceeds that of class II defensins (Fig. 6). Therefore, the CTPP cannot be required simply for shielding positive charge, but is likely to have co-evolved with the cationic grip motif to specifically inhibit the strong interaction of these defensins with PI(4,5)P_2, as they are transported through the cell to the vacuole.

Interactions with cellular phospholipid membranes are a key component of the mechanisms of antimicrobial peptides. Here we show that the plant defensin NaD1 participates in a high affinity interaction with PI(4,5)P_2 and disrupts membrane bilayers through a mechanism that does not conform with other models for membrane permeabilising peptides. A new model is therefore needed for the mechanism of membrane permeabilisation by NaD1 and other solanaceous class II defensins. We have demonstrated that NaD1 is attracted to negatively charged phospholipid membranes. The association and dissociation of NaD1 with the bilayer are at equilibrium and the disorder in the membrane induced by NaD1 binding is reversible. When NaD1 encounters a patch containing PI(4,5)P_2 the defensin and phospholipid form a tight complex and these defensin–lipid complexes oligomerise and cause a massive loss of order in the membrane, leading to membrane leakage via a mechanism distinct from the non-specific amphipathic AMPs. However, PI(4,5)P_2 is not found on the outer leaflet of plasma membranes. Although the reversible binding of NaD1 to membranes lacking PI(4,5)P_2 explains the initial interaction of NaD1 with the plasma membranes of target cells, the question of how the defensin traverses the plasma membrane to gain access

to PI(4,5)P_2 on the inner leaflet or in organellar membranes and exert its membrane disrupting activity remains to be determined. The cyclotide kalata B1 crosses cell membranes by both endocytosis and direct translocation after specific interaction with phosphatidyl ethanolamine and induction of membrane curvature [35]. Perhaps a similar situation exists for NaD1, where multiple mechanisms contribute to transit the defensin into the cytoplasm where it subsequently forms complexes with PI(4,5)P_2 on the inner leaflet of the plasma membrane. Alternatively, given that NaD1 partially penetrates the bilayer even in the absence of PI(4,5)P_2, this may be sufficient to allow contact with PI(4,5)P_2 molecules on the inner leaflet. The high affinity of NaD1 for PI(4,5)P_2 drives the rapid association of protein with cognate lipid and the corresponding amplification of membrane disruption.

Blebbing of cancer cell membranes is observed in the anti-tumor activity of NaD1 [4]. The mechanism described above for liposomes likely contributes to this. Blebbing is not observed in fungal cells but instead the membrane is permeabilised with no detectable changes in ultrastructure [25]. This is attributed to the presence of the fungal cell wall, which restricts the mobility of the membrane. Inability of the plasma membrane to adapt to the mechanical stress induced by NaD1 is likely to lead to localised perturbations in the membrane, that permits the entry and exit of molecules. In summary, membrane disruption by NaD1 and other solanaceous class II defensins is unique and dependent on the presence of PI(4,5)P_2. Further investigation will reveal how well the model systems employed here represent the activity of defensins in biological systems.

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