An oomycete NLP cytolysin forms transient small pores in lipid membranes

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Microbial plant pathogens secrete a range of effector proteins that damage host plants and consequently constrain global food production. Necrosis and ethylene-inducing peptide 1–like proteins (NLPs) are produced by numerous phytopathogenic microbes that cause important crop diseases. Many NLPs are cytolytic, causing cell death and tissue necrosis by disrupting the plant plasma membrane. Here, we reveal the unique molecular mechanism underlying the membrane damage induced by the cytotoxic model NLP. This membrane disruption is a multistep process that includes electrostatic-driven, plant-specific lipid recognition, shallow membrane binding, protein aggregation, and transient pore formation. The NLP-induced damage is not caused by membrane reorganization or large-scale defects but by small membrane ruptures. This distinct mechanism of lipid membrane disruption is highly adapted to effectively damage plant cells.

**RESULTS**

**Plant sterols and low-salt concentration promote the binding of NLP<sub>Pya</sub> to membranes**

We generated model lipid vesicles containing tobacco leaf GIPCs and equimolar mixture of three most abundant plant sterols, β-sitosterol, campesterol, and stigmasterol (12, 13), and first assessed how lipid composition affects the binding of NLP<sub>Pya</sub>. NLP<sub>Pya</sub> did not bind to 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) (9) or the POPC:sterol bilayer (fig. S1A). However, NLP<sub>Pya</sub> binding correlated with the increasing amount of GIPCs in the lipid membranes, and the presence of sterols strengthened the interaction (Fig. 1A and fig. S1). We then generated stable supported lipid bilayers (SLBs) (fig. S2) and performed the titration experiment using the quartz crystal microbalance (QCM) assay (fig. S1B). The presence of sterols substantially increased the affinity of NLP<sub>Pya</sub>, yielding a dissociation constant (K<sub>d</sub>) of 162 ± 14 nM for POPC:GIPC:sterols 3:4:3 (molar ratio, all lipid mixtures) compared to 2.3 ± 0.7 µM for POPC:GIPC 6:4 (values are means ± SEM of the fit). The negligible concentration can affect molecular processes at the plant plasma membrane (10, 11). Plant plasma membranes are extremely complex and remain poorly characterized in terms of composition and structure (12). Besides GIPCs, which comprise up to 40 mole percent (mol %) of plant lipids and are located in the outer membrane leaflet (13), plant membranes also contain substantial amounts of sterols (~30 mol %) (14, 15). GIPCs in the presence of plant sterols were reported to promote formation of lipid phases and be enriched in liquid-ordered domains (15).

Known NLPs share a conserved three-dimensional structures (7, 16, 17). They are single-domain proteins with a central β sandwich, which is on one side surrounded by three α helices and on the opposite side by three flexible loops. Their negatively charged GIPC-binding cavity contains a bound divalent cation (7, 9). In this study, we used the structurally and functionally well-characterized cytolsin NLP<sub>Pya</sub> from the phytopathogenic oomycete *Pythium aphanidermatum* (7, 9) to investigate how NLP disrupts lipid membranes.
changes in energy dissipation during the titration experiments (fig. S3A) suggest that the interaction is surface-driven, with no measurable restructuring of the membranes even at high (40 μM) protein concentrations (fig. S3B).

Liposome sedimentation assays with increasing [NaCl] further revealed that the interaction of NLP_{Pya} with GIPC-containing liposomes was highly salt dependent and thus predominantly electrostatically driven (Fig. 1, C and D). This was independently confirmed by surface plasmon resonance (Fig. 1E). Furthermore, as opposed to its slow dissociation from GIPC-containing membranes due to specific head group interactions (Fig. 1E), the binding of NLP_{Pya} to control, negatively charged 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-(1’-rac-glycerol) (POPG)–containing bilayers in low salt was easily reversed (fig. S4).

NLP_{Pya} resides on the membrane surface

We next explored the binding mode of NLP_{Pya} (Protein Data Bank ID: 3GNZ) to POPC:GIPC 1:1 membranes with all-atom molecular dynamics (MD) simulations. We positioned the GIPC-binding cavity over the GIPC head group and the three loops in contact with the membrane surface, as the loop residue tryptophan at position 155 (W155) is required for cytotoxic activity (9). After relaxing this binding pose for 1 μs, we performed enhanced sampling meta-dynamics simulations to accelerate the exploration of other possible NLP_{Pya} binding poses and estimate their relative free energies (18). After 1 μs of simulation, NLP_{Pya} adopted three distinct binding modes, all positioning the protein on the membrane surface and exhibiting the carboxylic acid of GIPC’s glucuronic acid moiety stably bound with the Mg^{2+}-ion placed within the NLP_{Pya} cavity (fig. S5). In the most stable pose, the C-terminal helix of NLP_{Pya} lies on top of the membrane (minimum a in Fig. 2, A and B), whereas in the other two metastable minima, b and c (Fig. 2A), the C-terminal helix more deeply penetrates into the GIPC head group region (Fig. 2C), or W155 more closely interacts with the GIPC head groups (Fig. 2D), respectively. The simulations strikingly reveal a rough free-energy surface underlying the binding of NLP_{Pya} to the membrane model, in which the free energy required to leave the main minimum is 7.0 ± 0.6 kcal/mol and all metastable states lie within 5.0 ± 0.6 kcal/mol above it. Consistent with the [NaCl] dependence of the NLP_{Pya}-GIPC interaction (Fig. 1, C to E), a calculation of the interaction energies between the membrane and NLP_{Pya} reveals that the electrostatic interactions mostly contribute to stabilizing the protein-binding pose, although hydrophobic interactions also form (fig. S5 and table S1). Several persistent hydrogen bonds are established between the GIPC head groups and NLP_{Pya} residues from the loops surrounding the binding cavity and from the C-terminal helix (fig. S5 and table S2). Thus, our MD simulations suggest how NLP_{Pya} binds strongly to GIPC lipids and predict partial insertion into the outer head group region.

We used neutron reflectometry (NR) to experimentally test the MD simulations, as this approach can yield detailed penetration profiles for membrane-bound proteins (19). Membrane interactions were examined independently with natural hydrogenous (h-NLP_{Pya}) and deuterated (d-NLP_{Pya}) proteins (fig. S6). d-NLP_{Pya} was used to...
resolve the protein within the hydrogen-rich, hydrophobic, membrane core of SLBs. For the POPC:GIPC 6:4 bilayer, NR revealed that a ~29-Å-thick layer of h-NLP Pya, corresponding to the thickness of a monomer, formed on the outer membrane surface (Fig. 2E, fig. S7, and table S3). Consistent with the MD simulations (Fig. 2, B to D, and fig. S5), the protein was not detected within the membrane core, with only a minor component in the outer bilayer head group region (Fig. 2E and table S3). The interaction of d-NLP Pya with the bilayer was similar to that of h-NLP Pya, creating a ~26-Å-thick layer on the membrane surface (fig. S8A and table S3). The interaction of d-NLP Pya with GIPC in the Ld phase, then accumulated at the phase boundary, and last formed tightly packed clusters on the GIPC-rich Lo domains (Fig. 3, B and C; figs. S11 and S12; and movies S1 to S3), similar to the ordered assemblies observed with TEM (Fig. 3A). Clusters of 9 ± 2 nm in diameter and 3 ± 1 nm in height, with a central depression, were observed on the membranes (Fig. 3, D to F). The thickness of the protein layer on the membrane thus agrees with the MD simulations and NR data (Fig. 2, A to E, and table S3). The large standard deviation, especially in cluster width (Fig. 3F), might be due to the variation in cluster stability and mobility, as assembled proteins were easily deformed or displaced by the HS-AFM probe because of their shallow binding to the membrane (Fig. 3D and fig. S13). During the binding and assembly of NLP Pya, the GIPC-rich Lo domains neither merged nor reorganized, even after full coverage with NLP Pya (figs. S11 and S12 and movies S1 and S3).

NLP Pya induces formation of transient pores permeable for small molecules

We then used a planar lipid bilayer approach and measurements of ionic currents (20) to assess the NLP Pya-induced damage of the membrane. NLP Pya-induced membrane openings (Fig. 4A) differ from discrete membrane openings induced by typical pore-forming
its membrane-disrupting capacity (Fig. 4C). NLP Pya-induced per-

$$\tau \text{ 4.4 } \pm \text{ 3.4 min} \text{ (Fig. 5, C and D, and fig. S14B). The leakage (} \text{leak}) \text{ of } \text{NLP Pya-A488 molecules out of the vesicle due to membrane permeabilization. This time lag (} \tau \text{bind}) \text{ between the saturation of the NLP Pya-A488 signal on the membrane and the onset of membrane permeabilization was } \text{4.4 } \pm \text{ 3.4 min (Fig. 5, C and D, and fig. S14B).}$$

To estimate the size of the NLP Pya pore, we imaged giant unilamellar vesicles (GUVs) along with differently sized fluorescent dextrans (FDs). NLP Pya induced pores permeable to 4-kDa FD (≈2.8 nm in diameter), but not 10- and 70-kDa dextrans (4.6 and 12 nm in diameter, respectively) (Fig. 5A). NLP Pya membrane attachment did not affect GUV morphology, as the vesicles neither dis-integrated nor collapsed during the time frame of the experiments.

Last, the binding of Alexa Fluor 488–labeled NLP Pya (NLP Pya-A488) to GIPC-containing GUVs and the subsequent membrane permeabilization were observed in real time in a custom-developed micro-fluidic diffusion chamber (fig. S14A) (22). GUVs in low [NaCl] filled with Alexa Fluor 594 (A594) were monitored with time-lapse microscopy using two fluorescence channels (Fig. 5B and movie S4). The NLP Pya-A488 signal on the membrane was coupled to the increasing concentration of NLP Pya-A488, diffusing into the chamber, and exhibited a sigmoidal shape with a binding time ($$t_{\text{bind}}$$) of 6.7 ± 3.3 min (Fig. 5, C and D, and fig. S14B). Several minutes later, the A594 signal in the vesicles decreased, indicating the leakage of A594 molecules out of the vesicle due to membrane permeabilization. This time lag ($$t_{\text{lag}}$$) between the saturation of the NLP Pya-A488 signal on the membrane and the onset of membrane permeabilization was 4.4 ± 3.4 min (Fig. 5, C and D, and fig. S14B). The leakage ($$t_{\text{leak}}$$) of A594 out of the vesicle was a relatively fast process (1.7 ± 1 min). When the NLP Pya-A488 solution was washed from the chamber, the signal on the membranes remained unchanged (fig. S14C). No binding of the protein was observed on pure POPC GUVs (fig. S14D).

**DISCUSSION**

Here, we provide insight into the unique molecular mode of action of membrane damage for a member of a large family of plant pathogen virulence effectors (7, 8). On the basis of our results, we suggest that NLP Pya-induced membrane damage occurs in several successive steps (fig. S16) composed of a predominantly electrostatically driven, initial association with membrane lipid receptors, GIPCs (Figs. 1 and 2), followed by protein clustering on the membrane plane (Fig. 3) and formation of transient heterogeneous pores that are permeable to small molecules (Figs. 4 and 5). Sterols may affect the availability of the GIPC head groups for NLP Pya binding, similar to cholesterol that modulates conformation of glycosphingolipid head groups (23), and allow the formation of tightly clustered NLP Pya aggregates on L_o domains (Fig. 3).

An important advantage of the real-time observations and the analysis of the experiments in the microfluidic system is that they allow for the testing of different kinetic models of the mechanisms of membrane permeabilization (text S1 and fig. S15). In the simplest model, it can be assumed that membrane permeability is proportional to the concentration of membrane-bound NLP Pya. Modeling revealed that regardless of the value of the permeability constant, it is not possible to reproduce the observed time lag between the NLP Pya saturation on the membrane and the onset of the solute leakage out of the vesicles within this model (fig. S15A). The observed time lag hints that membrane permeabilization is linked to a stochastic process on the membrane. For example, a stochastic formation of a large membrane rupture could lead to the observed evolution of the fluorescent signals in Fig. 5C. However, such a membrane rupture would also lead to a leakage of large molecules, which is not consistent with our observations (Fig. 5A). This sigmoidal kinetic process, with a time lag, is reminiscent of those observed in various processes of protein aggregation, such as formation of functional oligomers of
pore-forming toxins (24) or the growth of amyloid-β or α-synuclein aggregates related to neurodegenerative diseases (25). According to a minimal two-step model that was proposed for modeling these phenomena (26), membrane-bound NLP$_{Pya}$ undergoes a slow nucleation process that then triggers a fast protein clustering. Assuming that membrane permeability is proportional to the amount of clustered proteins (as if, for example, each protein cluster comprised a fixed density of small pores that allow the leakage of small molecules), we were readily able to reproduce the observed behavior (Fig. S15B). The observed time lag between the NLP$_{Pya}$ saturation on the membrane and the membrane permeabilization (Fig. 5, C and D) thus indicates that there is a step that involves association of NLP$_{Pya}$...
at the surface of the membrane and agrees with TEM, HS-AFM, and the measurements of electrical conductivities (Figs. 3 and 4).

While functionally reminiscent of bacterial membrane damage induced by cationic peptides via a carpet mechanism (27), NLP\text{Py}a membrane damage differs because of its lipid specificity. β-barrel pore-forming proteins often form prepore intermediates that are distinct in height from the inserted final pores (21, 28). Conversely, no significant changes in the thickness of the protein layer on the membrane were observed (Figs. 2 and 3), implying that NLP\text{Py}a does not undergo substantial conformational changes or deeper insertion into the membrane to induce membrane damage. The current noise observed during our planar bilayer measurements indicates that NLP\text{Py}a-induced membrane damage is in the form of transient ruptures that are permeable to small molecules (Figs. 4 and 5). The NLP\text{Py}a-induced permeabilization may be formed via shallow bilayer penetration and local GIPC reorganization due to numerous concomitantly (NLP Pya -induced permeabilization may be formed via shallow bilayer ruptures that are permeable to small molecules (Figs. 4 and 5). The NLP\text{Py}a-induced membrane disruption must thus for plant pathogens. NLP Pya -induced membrane disruption must thus for combating NLP-producing microbial pathogens (34).

MATERIALS AND METHODS

Lipid material

The lipids POPC, POPG, DOPC, DPhPC, β-sitosterol, and stigmasterol were supplied from Avanti Polar Lipids (Alabaster, AL, USA). β-Sitosterol for HS-AFM experiments and campesterol were purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA). Rhodamine-labeled 1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine (rhodamine-DHPE) was purchased from Thermo Fisher Scientific (USA).

Expression and purification of NLP\text{Py}a

The NLP\text{Py}a protein was expressed and purified as described previously (7, 9, 35). The production of deuterated d-NLP\text{Py}a for NR experiments followed the same protocol, with the following modifications. The initial adaptation of \textit{Escherichia coli} BL21 (DE3) to D\textsubscript{2}O (Cambridge Isotopes Lab Inc.) was achieved with four sequential precultures over 3 days with increasing D\textsubscript{2}O levels in M9 medium supplemented with 1 mM MgSO\textsubscript{4}, 0.1 mM CaCl\textsubscript{2}, 0.1 mM MnCl\textsubscript{2}, 50 μM ZnSO\textsubscript{4}, 50 μM FeCl\textsubscript{3}, vitamins (pyridoxine, biotin, d-pantothenic acid, folic acid, choline, niacinamide, riboflavin, and thiamine), ampicillin (100 μg/ml) as a selection marker, and 0.2% (w/v) glucose (nondeuterated) as the sole carbon source. LB H\textsubscript{2}O medium was inoculated with clones from a fresh transformation plate (9) and incubated at 37°C and 200 rpm (preculture 1). In the evening, the supplemented M9 H\textsubscript{2}O medium was inoculated with preculture 1 and incubated overnight (preculture 2). The next day, supplemented M9 50% D\textsubscript{2}O was inoculated with preculture 2 (preculture 3) and used in the evening to inoculate supplemented M9 D\textsubscript{2}O (preculture 4). The next morning, preculture 4 was used to inoculate ~1.8 liters of M9 D\textsubscript{2}O. When the culture reached OD\textsubscript{600} (optical density at 600 nm) of ~0.8, 0.5 mM isopropyl-β-D-thiogalactopyranoside was added to induce protein production. Cells were cultivated at 26°C and 180 rpm for 15 hours. The protein was purified with ion-exchange chromatography on a gravity flow nickel affinity column (Ni-NTA Superflow, Qiagen) (35). Protein-containing fractions were pooled and dialyzed against D\textsubscript{2}O.

The purity of the nondeuterated (h-NLP\text{Py}a) and deuterated (d-NLP\text{Py}a) protein preparations was verified by SDS–polyacrylamide gel electrophoresis (SDS-PAGE; fig. S6A). The activities of the proteins were identical, as measured by infiltration assay (fig. S6B). The proteins also had comparable circular dichroism and temperature melting profiles (fig. S6, C and D) and bound to GIPC-containing vesicles in a similar manner (fig. S6E).

Preparation of GIPCs

GIPCs were extracted and purified as previously described (9, 36). Briefly, tobacco (\textit{Nicotiana tabacum}) leaves were blended with cold 0.1 N aqueous acetic acid and filtered through miracloth. The slurry was extracted with hot 70% ethanol/0.1 N HCl. The pellet was washed with cold acetone and diethyl ether to yield a whitish precipitate, which was then dissolved in tetrahydrofuran:methanol:water (4:4:1, v:v:v) containing 0.1% formic acid. Dried precipitate was submitted to a butan-1-ol:water (1:1, v:v) phase partition. Residue from the upper GIPC-containing butanol phase was dissolved in tetrahydrofuran:methanol:water (4:4:1, v:v:v) containing 0.1% formic acid. GIPCs were characterized by matrix-assisted laser desorption/ionization mass spectrometry (36), and their mass was estimated from their dry weight. To test 40 μM NLP\text{Py}a in the QCM experiment, SLB was prepared from tomato GIPCs, purified in the same way as described above.

Liposome preparation

Liposomes were prepared by mixing POPC or DOPC with tobacco GIPCs and/or an equimolar mixture of the sterols β-sitosterol, campesterol, and stigmasterol. The final concentration of sterols in the lipid mixture was kept constant at 30 mol % (15). For some lipo- some sedimentation and surface plasmon resonance experiments, lipid mixtures of POPC and POPG were used. Multilamellar vesicles (MLVs) were prepared by hydration of thin lipid films in appropriate warm buffer and vortexing of the lipid suspensions in the presence of glass beads. Small unilamellar vesicles (SU Ve) were obtained by sonication of MLVs on ice for a total of 30 min, with 10-s on-off cycles at 40% amplitude using Vibracell Ultrasonic Disintegrator VCX 750 (Sonics and Materials, Newtown, USA). Large unilamellar vesicles (LUVs) were prepared from MLVs by
several freeze/thaw cycles and subsequent extrusion through polycarbonate filters with 50- or 100-nm pore size using a two-syringe extruder (NanoSizer Extruder, T&T Scientific, USA). The vesicle size distribution was routinely checked by dynamic light scattering (Zetasizer Nano-ZS, Malvern Instruments, UK).

**Liposome sedimentation assay**

Liposome sedimentation assay was performed as previously described (9, 17), with some modifications. Briefly, NLP$_{Py}$ (0.0625 mg/ml) was incubated with MLVs (5 mM) composed of either POPC, GIPCs, and/or sterols or of POPC and POPG for 30 min and 600 rpm at room temperature. The effects of sterols and salt on protein binding were tested in 20 mM MES and 150 mM NaCl (pH 5.8) and in 20 mM MES and 2 mM EDTA (pH 5.8) containing 0, 50, 150, or 500 mM NaCl. The mixture was centrifuged, and the liposomes were washed twice with the same buffer and subjected to SDS-PAGE, followed by Coomassie Brilliant Blue staining. Protein bands were statistically analyzed after densitometric quantification using an image analyzer system [ImageJ 1.29x, National Institutes of Health (NIH), USA]. Each value represents the average of three independent experiments.

**QCM measurements**

SUVs (2 mg/ml) were diluted to 0.15 mg/ml in buffer [20 mM MES, 150 mM NaCl (pH 5.8), and 5 mM CaCl$_2$], which was added to promote the rupture of vesicles to form SLBs (37). The impedancen-based QCM Z-500 instrument (KSV Instruments Ltd., Helsinki, Finland) was used with its own peristaltic pump system (Ismatec/ Cole-Parker GmbH, Wertheim, Germany). Vesicles were flown over the QCM crystals (Q-Sense Inc./BiolinScientific, Västra Frölunda, Sweden) for 5 to 10 min at flow speeds of 250 μl/min. After 5-min injections of buffer and buffer with 5 mM EDTA, two consecutive injections of ultrapure water were performed to induce osmotic shock to rupture the remaining vesicles on the surface. After reducing the flow speed to 75 μl/min, increasing concentrations (50, 150, 250, 500, 1000, and 2000 nM) of NLP$_{Py}$ were sequentially injected for 10 min. Each injection was followed by a 10-min dissociation phase. Sensors were cleaned in situ with sequential injections of 20 mM CHAPS, 2% (v/v) Hellmanex, 50% (v/v) ethanol, and ultrapure water.

Data were analyzed with in-house Python scripts using the rigid (Sauerbrey) layer model. When required, baseline corrections for the data were performed using OriginPro software (v. 2018b, OriginLab Corp., Northampton, MA, USA), and the data were interpolated to set a common time axis. Kinetic titration data corresponding to the hydrated surface mass density were fitted using the one-site kinetic model in TraceDrawer software (v. 1.9.1, Ridgeview Instruments AB, Vänge, Sweden).

**Surface plasmon resonance**

The binding of NLP$_{Py}$ to POPC:GIPC:sterols 3:4:3 (n:n:n) lipid bilayers was monitored with Biacore X100 (GE Healthcare) and the QCM crystals (Q-Sense Inc./BiolinScientific, Västra Frölunda, Sweden) was used with its own peristaltic pump system (Ismatec/ Cole-Parker GmbH, Wertheim, Germany). Vesicles were flown over the QCM crystals (Q-Sense Inc./BiolinScientific, Västra Frölunda, Sweden) for 5 to 10 min at flow speeds of 250 μl/min. After 5-min injections of buffer and buffer with 5 mM EDTA, two consecutive injections of ultrapure water were performed to induce osmotic shock to rupture the remaining vesicles on the surface. After reducing the flow speed to 75 μl/min, increasing concentrations (50, 150, 250, 500, 1000, and 2000 nM) of NLP$_{Py}$ were sequentially injected for 10 min. Each injection was followed by a 10-min dissociation phase. Sensors were cleaned in situ with sequential injections of 20 mM CHAPS, 2% (v/v) Hellmanex, 50% (v/v) ethanol, and ultrapure water.

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**Circular dichroism spectroscopy**

Far-ultraviolet circular dichroism spectra of h-NLP$_{Py}$ and d-NLP$_{Py}$ (0.25 mg/ml) were recorded using a 0.1-cm path length quartz cuvette on a Chirascan spectrometer (Applied Photophysics) in ultrapure water and D$_2$O, respectively. Spectra were measured in a 200- to 250-nm wavelength range with a step size of 0.5 nm and an integration time of 1 s at 20°C. Each spectrum represents the average of 10 scans. Spectra were baseline-corrected, smoothed, and processed with the Chirascan software. Spectral units were expressed as the mean molar ellipticity per residue.

**Thermal stability assay**

The thermal stability of h-NLP$_{Py}$ and d-NLP$_{Py}$ (0.1 mg/ml) was measured with the differential scanning fluorimetry (ThermoFlour) assay using 10x SYPRO Orange dye (Thermo Fisher Scientific). The thermal stability of proteins was tested in 20 mM MES (pH 5.8) prepared in ultrapure water (for h-NLP$_{Py}$) or D$_2$O (for d-NLP$_{Py}$). Samples were subjected to a temperature gradient from 25°C to 95°C at steps of 1°C/min. Temperature melting profiles were acquired with LightCycler 480 (Roche). Melting temperatures ($T_m$) were determined using the Boltzmann function in Origin 8.1 (OriginLab). Each $T_m$ value represents the average of six repeats in three independent experiments.

**Infiltration assay**

The infiltration assay was performed on tobacco plants (N. tabacum “White Burley”). h-NLP$_{Py}$ and d-NLP$_{Py}$ (50 μl of 500 nM) in ultrapure water and D$_2$O, respectively, were infiltrated abaxially into tobacco leaves using a syringe without a needle. Lesion formations were examined 24 hours after infiltration.

**MD simulations**

Starting from the experimentally determined structure of NLP$_{Py}$ with a Mg$^{2+}$ ion trapped in the central cavity (Protein Data Bank ID: 3GNZ) (7), we manually docked the protein onto the GIPC sugar head group (9) in the center of a POPC:GIPC 1:1 membrane bilayer so that the sugar head group (i.e., the glucuronic acid and glucosamine moieties) was inside the Mg$^{2+}$-binding cavity. The protein was initially placed on the membrane so that its tryptophane at position 155 (W155), which was demonstrated to be important for the cytolytic activity of NLP$_{Py}$ (9), was near a preequilibrated membrane surface. The membrane was built by the membrane generator MemGen (40). The symmetric membrane bilayer contained 80 GIPC molecules and 80 POPC molecules. The system was solvated by a layer of 30 Å of water molecules in the z direction, leading to a total of 61,461 atoms.

The system topology was built with the teap module of the amber tools 18 using the Amber parm99SB parameters for the protein (41) and lipid17 parameters for POPC. GIPC was parametrized according to the following procedure: The GIPC molecule was...
geometry-optimized with the density functional theory with the B3LYP exchange correlation function and the 6-31G* basis set using the Gaussian09 program (42). Charges were calculated according to the Restricted Electrostatic Potential (RESP) approach with the antechamber module of Amber18 tools (43). The other parameters were determined with the antechamber tool of Amber18 using the general amber force field parameters for organic molecules (44). The protonation states of the residues were determined with the Propka web server (45). The water molecules were described using the TIP3P water model (46). Charge neutrality was achieved by the addition of 76 Na+ ions described with the Joung and Cheatham parameters (47). The Mg2+ ion in the binding cavity was described with the Allner et al. parameters (48). The system topology was then converted to the GROMACS format with the acpype software (49).

Preliminary energy minimization was done with the steepest descent algorithm. An initial equilibration of the membrane was performed for 100 ns, keeping the protein atoms harmonically restrained with a force constant of 1000 kJ/mol nm², until a constant value (92 Å × 92 Å × 151 Å) of the simulation box size was reached. Constraints were then released, and the system was slowly thermalized to the target temperature of 300 K during a 10-ns MD. Last, the system was further equilibrated for 1 μs.

For all MD simulations, the pressure was kept to equilibrium value with the Parrinello-Rahman barostat (50), while the temperature was controlled with the velocity rescale thermostat (51). MD simulations were performed using GROMACS 2018.2 (52) and an integration time step of 2 fs. All covalent bonds involving hydrogen atoms constrained with the LINCS algorithm (53). The particle mesh Ewald scheme (54) was used to account for electrostatic interactions.

Well-tempered metadynamics simulations (55) were performed on the equilibrated trajectory using the plumed 2.4.3 plugin (56). In these simulations, two collective variables (CVs) were used, describing the distance of the tryptophan at position 155 (W155) and C-terminal helix centers of masses to the center of mass of the membrane (CV1 and CV2, respectively) to account for their penetration into the membrane bilayer. CV1 was defined as the z projection of the distance of the center of mass of W155 to the center of mass of the membrane, and CV2 was defined as the distance of the center of mass of the C-terminal helix of NLP Pya (residues 200 to 210) to the center of mass of the membrane. In the simulations, we used Gaussian hills of heights of 1.2 kJ/mol and sigma of 0.08 Å and 0.1 Å for CV1 and CV2, respectively. The bias factor was 30, and the Gaussian deposition rate was one every 500 steps.

The error of the free-energy profile was calculated as the SD of different time averages of CVs from different simulation blocks. The number of blocks was chosen using the block analysis technique to obtain uncorrelated data, i.e., looking at the average error as a function of the block sizes until reaching a plateau.

Interaction energies were calculated with the g_energy tool, while the root mean square deviation was monitored with the gmx_rms tool of GROMACS 2018.2. The hydrogen (H)–bonds were calculated with AMBER’s cpptraj tool. The fatslim program (57) was used to calculate the tail thickness and area per lipid of the membrane: 31.8 ± 0.28 Å and 59.6 ± 0.4 Å², respectively. This is in good agreement with the measured values reported in table S3.

**Neutron reflectometry**

Neutron reflectometry (NR) measurements were performed with the white beam INTER reflectometer (58) at the ISIS Spallation Neutron Source, Rutherford Appleton Laboratory (Oxfordshire, UK), which uses neutron wavelengths of 1 to 16 Å. The reflected intensity was measured at glancing angles of 0.7° and 2.3°. Reflectivity was measured as a function of the momentum transfer: \( Q_x = (4π \sin \theta)/\lambda \), where \( \lambda \) is the wavelength and \( \theta \) is the incidence angle. Data were obtained at a nominal resolution \((dQ/Q)\) of 3.5%. The total illuminated sample length was ~60 mm. Liquid flow and liquid chromatography systems were set up as previously described (19).

Briefly, solid liquid flow cells were placed onto the instrument sample position and connected to instrument-controlled high-performance liquid chromatography (HPLC) pumps (Kauer Smartline 1000), which controlled changes in the solution, i.e., isotopic contrast as well as counter-ion concentration and type in the flow cells.

During the NR measurements, the membrane-free, clean, silicon surfaces were initially measured in 20 mM MES (pH 5.8) D2O and 20 mM MES (pH 5.8) H2O buffer solutions. NLP Pya (500 nM) in H2O buffer was then flushed into the solid liquid flow cell. The NR data in this contrast were used to assess the binding of both d-NLP Pya and h-NLP Pya to the membrane. Then, data were collected for the same set of contrasts for the proteo-membrane complex and for the membrane alone.

**NR data analysis**

NR data were analyzed using the RasCal fitting package (version 2014b, A. Hughes, ISIS Spallation Neutron Source, Rutherford Appleton Laboratory), which uses an optical matrix formalism (59, 60) to fit layer models of the interfacial structure to the experimental reflectivity data. The program is designed to support the simultaneous fitting of multiple sets for which parameters, such as the layer thickness and roughness (grading of either the bulk interface or interface between layers), are fully or partially fixed across multiple datasets, while the scattering length density (SLD) and derived values, such as volume fraction/component coverage, can be assumed to vary. The relationships between the SLDs, layer thickness, and lipid area per molecule were defined in the software’s “custom fitting” option. Models describing the interfacial layer structure between the silicone substrate (super phase) and buffered water (subphase) consisted of a SiO2 layer, inner bilayer head groups, bilayer tails, and outer bilayer head groups.

NR data describing NLPPy binding to GIPC-containing SLBs were analyzed by fitting eight sets of reflectometry data describing stages of the experimental process. These were, in order, data from the following: the bare silicon surfaces in two solution isotopic contrast conditions (D2O and H2O buffers), SLB before NLPPy binding in three solution isotopic contrast conditions (D2O, Si-MW, and H2O buffers), and membrane after NLPPy binding also in three solution isotopic contrast conditions (D2O, Si-MW, and H2O buffers).

The fitting parameters for the SiO2 layer were assumed to be the same across all datasets obtained under varying isotopic contrast conditions of the solutions. The reflectivity datasets obtained for the silicon surfaces without the presence of the bilayer at the bulk...
interface were used to constrain the fitting of the roughness of the silicon and surface SiO$_2$ layer. Roughness was modeled using a series of unroughened 1-Å layers (analogous to partitioning a curve for a midpoint Riemann sum) with an SLD that followed the gradient of the graded interface.

The lipid bilayer data were fitted to three parameters: the area per lipid molecule, explicitly associated water (with the lipid head group regions), and surface coverage. This strategy was useful for the membrane studies described here because it enabled the linking of the lipid head group and tail parameters, as they are part of the same molecule. This approach minimized the number of free parameters in the model and yielded useful quantities, such as hydration or area per molecule. The procedure was previously described in detail by Hughes et al. (61). Briefly, in the custom fitting script, the relationship between layer thickness, component volume, and area per molecule is defined as

$$\text{Thickness} = \frac{\text{Volume}}{\text{Area per molecule}}$$ (1)

Component volume, scattering length ($b$), and SLD are

$$\text{SLD} (p) = \sum b / \text{Volume}$$ (2)

The volume and scattering lengths of each of the interfacial components were known or calculated. As all the membranes studied had composite lipid compositions, the molecular volume and scattering lengths used were the sum of individual lipid components multiplied by their volume fraction in the vesicles used to deposit SLBs (table S3). By fitting the interfacial lipid area per molecule, the layer thicknesses were calculated within the software and used in the matrix calculation that was used to model the reflectivity profiles and fit these to the experimental data.

Upon the interaction of NLP$_{pya}$, the lipid component of the membrane was again fitted using an area per molecule approach but with the addition that the nonlipid component of the membrane was described as a ratio of protein and solution. This approach allowed the volume fraction of lipid, solution, and protein in the membrane to be resolved. Lipid area per molecule, protein, and solution content of the NLP$_{pya}$-bound membrane was fitted independently of the initially characterized bilayer. The substrate roughness and SiO$_2$ layer characteristics were constrained across all datasets. An additional layer was added to the NLP$_{pya}$-bound membrane, which described a layer of protein on the surface of the membrane. This layer was unconstrained, meaning that it could have a minimal thickness of 0 Å and a hydration between 0 and 100% solution (i.e., no protein). A single protein layer with asymmetric roughness on the membrane surface was found to adequately describe the protein distribution in all cases of NLP$_{pya}$ binding to the GIPC-containing membrane surfaces as was seen through optimal fitting of the experimental reflectometry data using this description of the protein component.

Error estimation was performed using Rascals Bayesian error estimation routines (62, 63), with the log-likelihood function described in terms of chi-squared (62, 63). Marginalized posteriors were obtained using a Delayed Rejection Adaptive Metropolis algorithm (64, 65), and the best-fit parameters were taken as the distribution maxima; the uncertainties presented here are from the shortest 65% confidence intervals of each distribution.

The layer thicknesses and component volume fraction parameters were either intrinsically fitted (such as the SiO$_2$ and protein components) or determined from the fitted area per molecule and hydration parameters (lipid component); the model fits were used to produce volume fraction versus distance profiles for the interfacial structural components. Monte Carlo Markov Chain (MCMC) Bayesian error estimation results from the model-to-data fits were used to determine ambiguity of each volume fraction versus distance profile and are shown as a line width across each component distribution. The volume fraction of an individual component was calculated in 1-Å increments from −10 to 110 Å across the solid/liquid interface (the silicon/silicon dioxide interface set as the zero position). The mean, lower, and upper 95% confidence interval bounds of each component distribution were determined for every 1-Å segment within the set range using the MCMC error estimation results; these confidence intervals were then used to produce a line width error region above and below the mean values. The water distribution was calculated as the remaining unoccupied volume and summed across the interface with the appropriate error propagation.

**Transmission electron microscopy**

LUVs composed of POPC:GIPC:sterols 1:6:3 in buffer [20 mM MES and 20 mM NaCl (pH 5.8)] were incubated with 500 nM NLP$_{pya}$ for 5 min at 20°C. The sample suspensions (4 μl) were then applied onto Formvar-coated, carbon-stabilized, and glow-discharged (EM ACE200, Leica Microsystems, Vienna, Austria) copper grids and contrasted with 1% (w/v) uranyl acetate (aqueous solution). Samples were imaged with a CM 100 transmission electron microscope (Philips, The Netherlands) at 80 kV, using an Orius SC200 camera and Digital Micrograph Suite software (Gatan, USA).

**HS-AFM imaging**

For HS-AFM imaging, SLBs were prepared by incubating 1.5 μl of 1 mM unilamellar vesicles [prepared from MLVs as described in the “Liposome preparation” section; buffer: 20 mM MES and 150 mM NaCl (pH 5.8)] on a 1.5-mm-diameter mica disk at 42°C for more than 30 min or at 55°C for less than 30 min; this was followed by extensive rinsing. HS-AFM imaging was performed with NANOEXPLORER (Research Institute of Biomolecule Metrology Co. Ltd., Tsukuba, Japan) using cantilevers with carbon nanofiber probes (BL-AC10FS-A2) provided by Olympus Co. (Tokyo, Japan). The cantilevers had a spring constant of 0.1 N/m and a resonance frequency of 450 to 500 kHz in aqueous medium. The scan direction for image acquisition was from left to right, and the scan rate was 0.5 frame/s. The SLB-deposited mica surface was imaged in 70 to 80 μl of buffer solution. Experiments were performed in 20 mM MES (pH 5.8) with or without 150 mM NaCl. After observing SLB, 10 μl of NLP$_{pya}$ was introduced into the imaging medium. The final concentration of NLP$_{pya}$ in the imaging medium was 300 nM or 10 μM.

**Planar lipid bilayer experiments**

Experiments were performed with the Nanion Technologies Orbit Mini set up and Ionera 100-μm MECA recording chips. Data were obtained with the Elements Data Reader v 3.8.3 (Elements) software package at a working range of 2 nA, sampling frequency of 20 kHz, and room temperature. DPhPC, GIPCs, and sterols were dissolved to 5 mg/ml in pure octane (analytical standard, Sigma-Aldrich Inc., St. Louis, MO, USA). Bilayers were formed by the manufacturer-provided brush. Recordings were performed in 120 μl of recording buffer [20 mM MES (pH 5.8), with 0.1 or 1 M NaCl] at −100 mV (for NLP$_{pya}$) or 50/100 mV (for the lysenin mutant RL1 pore) (21).
For the NLP Pya permeabilization experiments, freshly prepared another 75 min at 22°C and 0.5-V amplitude. GUVs sedimented l of ultrapure water at 55°C and covered film was rehydrated in 750 μl of 0.2 M sucrose and 5 μM A594 (molecular weight, 758.79 g/mol; Molecular Probes, Invitrogen, Thermo Fisher Scientific) solutions. The tubes were placed in a water bath at 65°C, and the electrodes were connected to an AC generator set to an amplitude of 8 V and a frequency of 10 Hz for the first 2 hours. After 2 hours, the water heating was switched off, and the settings were gradually lowered to 1 V and 1 Hz. The suspension of formed vesicles was cooled down to room temperature for ~30 min and then transferred into a plastic container together with an equal amount of an iso-osmolar glucose solution. The denser, sucrose-filled GUVs sank in the less dense sucrose/glucose mixture. The container with the GUV suspension was sealed, and the vesicles were used within 4 days.

GUVs [POPC:GIPC 1:1.2 (n:n)] for the dextran leakage experiments monitored by confocal laser scanning microscopy were prepared on a commercially available vesicle preparation station (Vesicle Prep Pro station, Nanion Technologies, Germany). Lipids were dissolved in chloroform:methanol 9:1 (v:v) to 2.5 mM, and the fluorescent probe rhodamine-DHPE was added for membrane labeling at a final lipid concentration of 0.5%. Lipid solution (10 μl) was placed on the conductive indium tin oxide–coated glass slide and dried under reduced pressure for at least 60 min. The lipid-covered slide was then transferred onto the vesicle preparation station, with dried lipid film positioned in the center of the O-ring. The lipid film was rehydrated in 750 μl of ultrapure water at 55°C and covered with another conductive slide. Electroformation was performed inside the station with an AC current of 3.5 V and 10 Hz applied across the slides for 165 min at 65°C. This procedure continued for another 75 min at 22°C and 0.5-V amplitude. GUVs sedimented spontaneously to the bottom of the conical falcon tube in ~4 days.

Confocal laser scanning microscopy
For the NLP Pya permeabilization experiments, freshly prepared GUV suspensions were mixed with 4-, 10-, or 70-kDa FDs (Sigma-Aldrich, St. Louis, MO, USA) and NLP Pya. The final concentrations of FDs and NLP Pya were 1 mg/ml and 500 nM, respectively. The mixture in ultrapure water was incubated for 30 min at room temperature. Images were then recorded on a Leica TCS SP5 laser-scanning microscope with a 63× oil-immersion objective (numerical aperture = 1.4), with fluorescence excitation/emission λ ranges of 480/500 to 530 nm (for FDs) and 543/644 to 650 nm (for rhodamine). Acquired images were analyzed with ImageJ software (ImageJ 1.29x, NIH, USA), and the permeabilization (%) of individual vesicles was calculated.

Microfluidic experiments
The real-time interaction of NLP Pya labeled with A488 (Molecular Probes, Invitrogen, Thermo Fisher Scientific) (NLP Pya-A488) with individual GUVs was studied with a microfluidic system with a diffusion chamber (fig. S14A). The design, fabrication, and characteristics of the microfluidic system were described previously (22). Briefly, the main microchannel in the microfluidic chip is 100 μm wide and 40 μm deep. The flow in the main channel can be hydraulically regulated by a simple adjustment of the height of the exit reservoir, and the solution that flows through the system can be easily exchanged in the entrance reservoir. The diffusion chamber (100 μm × 250 μm × 40 μm) is a dead-end channel extending sideways from the main channel. The chamber is essentially flow-free, and therefore, the exchange of solutes between the main channel and diffusion chamber only occurs via passive diffusion (66).

The microfluidic system was set on an inverted microscope (Nikon Eclipse Ti) equipped with an epifluorescence module (pE-300ultra, CoolLED, UK), optical tweezers (Tweez, Aresis, Slovenia; laser wavelength of 1064 nm), and a 60× water immersion objective (NIR APO; numerical aperture, 1.0). Experiments were recorded with a SCiO custom-made MatLab software.

A fresh microfluidic system was prepared for each set of experiments. The system was first flushed with an iso-osmolar glucose solution. A suspension of GUVs was introduced into the main channel, and the flow rate was set to approximately 10 μm/s. Suitable GUVs without visible membrane protrusions were selected from the main channel and individually transferred by optical tweezers into the diffusion chamber. Then, the system was flushed with an iso-osmolar solution without a fluorescent marker so that any fluorescent signals present originated only from A594 trapped inside GUVs. Last, an iso-osmolar solution of sucrose or glucose with 1 to 1.3 μM NLP Pya-A488 was introduced into the main channel and diffused toward GUVs in the diffusion chamber. Since the diffusion was slow, the protein concentration gradient along the chamber persisted throughout the experiment (the concentration at the rear end of the chamber was always lower than that in the main channel). The experiments were recorded in 3-s time lapses in transmitted light (to monitor membrane morphology) as well as in green (for NLP Pya-A488) and red (for A594) epifluorescence channels. As the protein diffused toward GUVs, some GUV movement was observed (movie S4) that could be diffusiophoretic in origin (68).

For each GUV, the intensities of three fluorescent signals were recorded: A594 in the vesicle interior, NLP Pya-A488 in the solution surrounding GUV, and NLP Pya-A488 on the GUV membrane. All fluorescent signals were corrected for interchannel cross-talk and fluorescent marker bleaching. The NLP Pya-A488 signal on the membrane was determined as the difference between the average intensity on the membrane minus the intensity in the surrounding solution. The A594 signal in the vesicle was calculated as the average A594 intensity in the vesicle interior.

To quantify the dynamics of NLP Pya binding and membrane permeabilization for each vesicle, the signals of NLP Pya-A488 on the membrane and A594 inside the vesicles were fitted by a logistic sigmoidal curve (fig. S14B). The time intervals of the NLP Pya-A488 signal increasing on the membrane (t band), the time lag between the saturation of the signal on the membrane and the onset of A594 leaking (t lag), and the time interval during which A594 molecules diffused out of the vesicle after the membrane has been permeabilized (t leak) were quantified by fitting sigmoidal curves to the fluorescence signals (dashed black curves on fig. S14B) and calculating the times when the fitted curves reached thresholds at 10 and 90%. t band for the
binding of the protein to the membrane was determined as the difference between the times when the sigmoid for NLP$_{Pys}$-A488 reached 10 and 90% of its final value. Here, it should be noted that the increasing of the NLP$_{Pys}$-A488 signal on the membrane is coupled to the diffusion of NLP$_{Pys}$ into the diffusion chamber, while the partition of NLP$_{Pys}$ from the surrounding solution to the vesicle membrane is rapid. $t_{\text{sig}}$ was determined as the difference between the time when the sigmoid for NLP$_{Pys}$-A488 reached 90% of its final value and the time when the sigmoid for A594 reached 90% of its initial value. $t_{\text{leak}}$ was determined as the difference between the time when the sigmoid for A594 decreased from 90 to 10% of its initial value. A total of 21 GUVs were analyzed in six independent experiments. In one of the analyzed GUVs, positioned at the very rear of the chamber where the NLP$_{Pys}$-A488 concentration was the lowest, A594 leakage was not observed during the entire experiment (~1 hour) despite visible NLP$_{Pys}$-A488 binding. In control experiments, in which GUVs were exposed to an iso-osmolar solution without the protein, GUVs remained stable in shape and without leakage of A594 for more than 2 hours.

**SUPPLEMENTARY MATERIALS**

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An oomycete NLP cytolysin forms transient small pores in lipid membranes

Katja Pirc
Luke A. Clifton
Neal Yilmaz
Andrea Saltalamacchia
Mojca Mally
Tina Snoj
Nada Žnidaršič
Marija Srnko
Jure Boršek
Petteri Parkkila
Isabell Albert
Marek Podobnik
Keiji Numata
Thorsten Nürnberg
Tapani Viitala
Jure Derganc
Alessandra Magistrato
Jeremy H. Lakey
Gregor Anderluh

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