Supporting Information

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SI Materials and Methods

Cloning and Expression of coreTAPL. A single-cysteine variant of human coreTAPL (Q9NP78 UniProt, amino acids 143–766) and a coreTAPL fusion construct were designed for expression in *Pichia pastoris*. Both constructs were flanked by EcoRI/XhoI restriction sites for cloning into the pPICZ-C vector (Invitrogen). The fluorescent version of coreTAPL (coreTAPL$_{mVenus}$) was C-terminally connected by a linker (STNLGSENLYFQGVAI-GGLAV) with the YFP derivative mVenus followed by a second linker (GLDAAGGGGSGGGGSLV) and a C-terminal His$_{10}$-tag. The single-cysteine mutant of coreTAPL, which shows the same activity as wt TAPL, is connected by a single serine with the C-terminal His$_{10}$. All four cysteines of coreTAPL were substituted by alanine or valine, and a single cysteine was introduced at position two. Transformation of the protease-deficient His$^+$ *P. pastoris* strain SMD1163 was performed by electroporation as described (1, 2) (1.5 kV, 25 μF, 400 Ω, Gene Pulser II; Bio-Rad), using 50–75 μg linearized DNA. Transformants with highest expression of both coreTAPL constructs were selected as described (1, 3, 4). Large-scale expression in a 7.5-L Labfors4 reactor (Infors HT) was performed by fed batch method (3). Cells were frozen in liquid nitrogen and stored at −80 °C.

Purification and Membrane Reconstitution of coreTAPL. Frozen *P. pastoris* cells were thawed on ice and mixed with 1/3 volume of breaking buffer [50 mM KH_2PO_4, 1 mM EDTA, 5 mM aminocaproic acid, 5% (vol/vol) glycerol, pH 7.4] and 1/3 volume of washed glass beads. Cells were disrupted by two times four cycles of 30-s shaking at 6 m/s in a FastPrep-24 system (MP Biomedicals). Cell debris was removed by centrifugation at 3,000 × *g* for 10 min. Membranes were harvested at 100,000 × *g* for 45 min at 4 °C and solubilized (5 mg/mL total protein) for 2 h at 4 °C in solubilization buffer [20 mM Na-Hepes, 500 mM NaCl, 2.5 mM benzamidine, 1 mM PMSF, 15% (vol/vol) glycerol, 1% DDM (wt/vol) (*n*-dodecyl-β-D-maltoside; Carl Roth), 20 mM imidazole, pH 7.5]. After centrifugation at 100,000 × *g* and 4 °C for 45 min, the supernatant was incubated with Ni-nitrilotriacetic acid (NTA) agarose (Qiagen) at an overhead rotor for 3 h at 4 °C. Subsequently, Ni-NTA agarose was loaded on a column and washed with 10 column volumes of Hepes buffer [20 mM Na-Hepes, 140 mM NaCl, 15% (vol/vol) glycerol, 0.05% DDM, pH 7.5] containing 20 mM imidazole and with 20 column volumes Hepes buffer containing 100 mM imidazole. CoreTAPL was eluted in Hepes buffer with 500 mM imidazole, which was removed afterward via a PD-10 column (GE Healthcare). CoreTAPL was concentrated on an Amicon spin concentrator (cutoff 100 kDa; Millipore), and the concentration of coreTAPL (ε_{280} = 45,270 L·mol^{-1}·cm^{-1}) and coreTAPL$_{mVenus}$ (ε_{515} = 92,200 L·mol^{-1}·cm^{-1}) was determined by absorption.

Reconstitution of coreTAPL was performed with liposomes composed of *E. coli* polar lipids and DOPC (Avanti Polar Lipids) in a 7:3 (wt/wt) ratio at a protein-to-lipid ratio of either 1:20 or 1:100 (wt/wt) as described (5). For liposome preparation, *E. coli* polar lipids and DOPC dissolved in chloroform were mixed in a 7:3 (wt/wt) ratio and a dry lipid film was generated by evaporation using a rotary evaporator. The lipid film was rehydrated to a lipid concentration of 10 mg/mL in reconstitution buffer [20 mM Na-Hepes, 140 mM NaCl, 5% (vol/vol) glycerol, pH 7.5] and treated for 30 min in a bath sonicator. Five times freezing in liquid nitrogen and thawing at room temperature followed by 11 times extrusion with an Avestin mini-extruder through a polycarbonate filter with 400-nm pore size (Avestin) ensured preparation of homogenous large unilamellar vesicles. Liposome size was determined by nanoparticle tracking analysis (NanoSight LM14; Malvern Instruments). To detect liposomes by DCFBA, the lipid-mimicking dye DiD ($\lambda_{ex/em}$ = 648/670 nm; Invitrogen) was added in a molar DiD:lipid ratio of 1:4,000 during liposome formation.

For reconstitution of TAPL, liposomes were diluted to 2.5 mg/mL of lipids and destabilized with Triton X-100 for 30 min. Detergent-destabilized liposomes were mixed with appropriate amounts of purified coreTAPL and incubated on an overhead shaker for 30 min at 4 °C. Detergent was removed by two times 40 mg/mL and two times 80 mg/mL (wet weight) polystyrene beads (Bio-Beads SM-2; Bio-Rad). Micro Bio-spin chromatography columns (Bio-Rad) were used to remove Bio-Beads. Proteoliposomes were harvested for 30 min at 270,000 × *g*, 4 °C, resuspended in reconstitution buffer [20 mM Na-Hepes, 140 mM NaCl, 5% (vol/vol) glycerol, pH 7.5] to a final lipid concentration of 5 mg/mL, and stored at −80 °C. For DCFBA experiments, proteoliposomes were extruded 11 times through a 200-nm polycarbonate filter.

Carbonate Extraction. To determine the reconstitution efficiency of TAPL, TAPL-containing proteoliposomes (70 μg coreTAPL$_{mVenus,}$ 1.4 mg lipid) were pelleted for 30 min at 270,000 × *g*, 4 °C, and resuspended in 500 μL ice-cold carbonate buffer (100 mM Na_2CO_3, pH 11.5). After incubation for 30 min on ice, proteoliposomes were collected for 30 min at 270,000 × *g*, 4 °C, and resuspended in 500 μL carbonate buffer containing 55% (wt/vol) sucrose. Carbonate buffer-treated proteoliposomes were layered on a sucrose step gradient (5% each) from 55% to 0% sucrose (500 μL each step) and centrifuged for 12 h at 200,000 × *g*, 4 °C, in a swing-out rotor (SW41 rotor; Beckman Coulter). After centrifugation, fractions (500 μL) were collected from top and analyzed by SDS/PAGE and immunoblotting.

TEV Cleavage. To determine the orientation of TAPL in liposomes, proteoliposomes (40 μg lipid) containing coreTAPL$_{mVenus}$ (2 μg coreTAPL) were incubated at 25 °C for 1 h in the presence or absence of 1 μg tobacco etch virus (TEV) protease. As control, proteoliposomes were solubilized with 1% Triton X-100, before TEV addition. After TEV treatment, samples were analyzed by SDS/PAGE and immunoblotting.

Peptide Labeling and Purification. Peptides were synthesized by solid-phase synthesis (Charité). For labeling, peptide RRYCKSTEL, dissolved in PBS (10 mM Na_2HPO_4, 2 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl, pH 6.5) supplemented with 20% (vol/vol) dimethylformamide to a concentration of 3.5 mM, was incubated for 1 h at room temperature with a 1.2 molar excess of ATTO488-maleimide or Atto655-maleimide (ATTO-TEC) dissolved in dimethylformamide. Labeled peptides were isolated by reversed-phase chromatography using a PerfectSil-300ODS-C18 column (MZ Analysentechnik) and a linear gradient from 5% to 100% (vol/vol) acetonitrile supplemented with 0.1% trifluoroacetic acid. Isolated fractions contained only labeled peptides as verified by mass spectrometry and reversed-phase HPLC. Concentration of peptides was determined by absorption (ε_{501} = 90,000 L·mol^{-1}·cm^{-1}, ε_{663} = 125,000 L·mol^{-1}·cm^{-1}).

Peptide Transport. Peptide transport was performed by using an ATP-regenerating system (ARS) or free ATP. In case of the ARS, 625 ng creatine kinase (Roche), 24 mM creatine phosphate disodium salt (Sigma-Aldrich), 3 mM $MgSO_4$, and 3 mM ADP were used (6). For transport, 50 μL of proteoliposomes (1 mg/mL lipid) were

incubated at 37 °C in transport buffer [20 mM Na-Hepes, 5% (vol/vol) glycerol, pH 7.5] containing 42.5 mM NaCl and peptides (RRYCKSTEL) labeled with ATTO488- ($\lambda_{ex/em}$ = 501/523 nm) or ATTO655-maleimide ($\lambda_{ex/em}$ = 663/684 nm) (ATTO-TEC). In case of measurements with only ATP instead of the ARS, the transport buffer contained 107 mM NaCl, 3 mM $MgCl_2$, and variable amounts of ATP. Transport (50 μL total volume) was started by addition of creatine kinase or ATP and stopped by adding 4 volumes of ice-cold stop buffer (PBS, 10 mM EDTA, pH 7.5). To determine background levels of fluorescence, creatine kinase or ATP were excluded from the assay.

For macroscopic analysis, samples were transferred to microfilter plates (MultiScreen plates, Durapore membrane, 0.65-μm pore size; Millipore) preincubated with 0.3% polyethylenimine. The filters were washed twice with 250 μL ice-cold stop buffer and incubated for 10 min with elution buffer (PBS, 1% SDS, pH 7.5) before quantification of the amount of transported peptide by a fluorescence plate reader (Polarstar Galaxy, BMG) at $\lambda_{ex/em}$ = 485/520 nm. Samples containing $coreTAPL_{mVenus}$ were heated at 95 °C for 10 min before quantification to exclude interference of mVenus fluorescence.

For DCFBA, proteoliposomes were pelleted at 270,000 × *g* for 20 min, washed with 3 mL ice-cold stop buffer (20 min, 270,000 × *g*), and suspended in reconstitution buffer [20 mM Na-Hepes, 140 mM NaCl, 5% (vol/vol) glycerol, pH 7.5] to a final lipid concentration of 0.5 mg/mL.

To address the possible influence of an electrochemical ion gradient on peptide accumulation, TAPL was reconstituted in the presence of phosphate buffer [140 mM KPi, 5% (vol/vol) glycerol, pH 7.5]. For transport, 50 μL of proteoliposomes (1 mg/mL lipid) containing coreTAPL (2.5 μg) were incubated in the presence of 1 μM of valinomycin or nigericin (Sigma-Aldrich), ARS, and 3 μM or 30 μM $RRYC^{ATTO488}KSTEL$ in phosphate buffer.

Transport Directionality. To investigate directionality of transport by DCFBA, proteoliposomes were prefilled with 3 μM $RRYC^{ATTO488}KSTEL$ through import at 37 °C for 20 min in transport buffer containing either 42.5 mM NaCl and ARS (6) or 107 mM NaCl and 3 mM Mg-ATP. The transport reaction was stopped, and the proteoliposomes were washed by ultracentrifugation at 270,000 × *g* for 20 min in 60 volumes of ice-cold reconstitution buffer to follow peptide exchange or of reversibility buffer [20 mM KPi, 140 mM NaCl, 5% (vol/vol) glycerol, pH 7.5] to detect reverse transport. Subsequently, proteoliposomes were resuspended to a final lipid concentration of 5 mg/mL in the respective buffer. The export of $RRYC^{ATTO488}KSTEL$ from the proteoliposomes was followed directly at the laser-scanning confocal microscope for 10 min at 37 °C. In case of counterflow transport, proteoliposomes were incubated in transport buffer containing 42.5 mM NaCl, ARS, and 60 μM unlabeled peptide (RRYQKSTEL). Transport reversibility experiments were carried out in reversibility buffer containing 3 mM ADP and 3 mM $MgCl_2$. As control of efflux, prefilled proteoliposomes were incubated for 10 min at room temperature with 9.8 μM of the pore-forming antimicrobial peptide alamethicin (Fermentek).

Prefilling of Proteoliposomes. For trans-inhibitory studies, TAPL containing proteoliposomes were prefilled with 3 mM unlabeled peptide (RRYQKSTEL) by three freeze−thaw cycles and two times washing with reconstitution buffer (20 min, 270,000 × *g*) (6). Proteoliposomes were resuspended in reconstitution buffer to a final lipid concentration of 5 mg/mL and used in peptide transport and ATPase studies. Empty control proteoliposomes were treated the same way, just without adding unlabeled peptide.

ATPase Assay. ATPase activity of reconstituted TAPL was measured by use of a malachite green-based colorimetric assay (7). Proteoliposomes (2 μg coreTAPL, 40 μg lipid) were incubated in ATPase buffer [20 mM Na-Hepes, 140 mM NaCl, 15% (vol/vol) glycerol/5 mM NaN_3, 1 mM Ouabain, 50 μM Na-EGTA, pH 7.5] in the presence of 3 mM Mg-ATP at 37 °C for 15 min. The reaction was stopped by addition of 20 mM H_2SO_4 and supplemented with malachite green solution [3 mM malachite green, 0.2% Tween 20, 1.5% (wt/vol) ammonium molybdate]. Absorption at 620 nm was measured after incubation at room temperature for 10 min.

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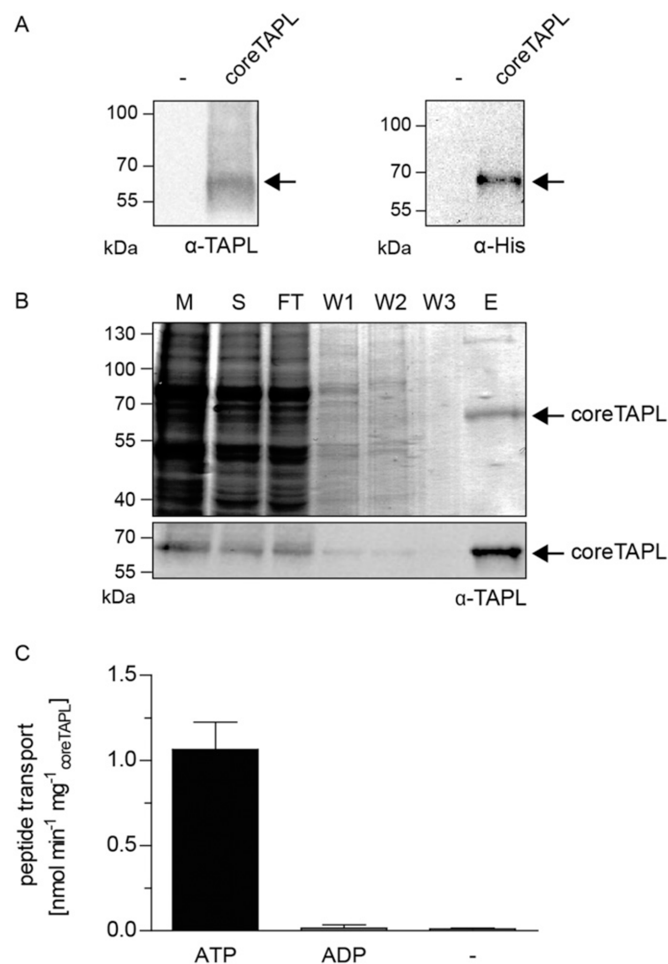

Fig. S1. Expression, purification, and functional reconstitution of coreTAPL. (*A*) Expression of coreTAPL in *P. pastoris* was demonstrated by SDS/PAGE and immunoblotting of crude membranes. Crude membranes of untransformed SMD1163-His^{+} cells (–) served as negative control. (*B*) Coomassie-stained SDS/PAGE and immunoblotting of coreTAPL purification. CoreTAPL in crude membranes (M) was solubilized in DDM (S) and purified by metal-affinity chromatography (FT, flow-through; W1-3, wash). Elution (E) was performed with 500 mM imidazole. (*C*) CoreTAPL (2.5 μg) was reconstituted in liposomes (50 μg lipid), comprised of a mixture of *E. coli* lipids and DOPC (7:3 mass ratio), and transport was performed for 15 min at 37 °C with 3 μM of RRYC^{ATTO488}KSTEL in the presence of 3 mM Mg-ATP or of Mg-ADP, or in the absence of nucleotide (–). The amount of transported peptide was analyzed by a fluorescence plate reader ($\lambda_{ex/em}$ = 485/520 nm). The transport assay was performed in triplicate with error bars indicating SD.

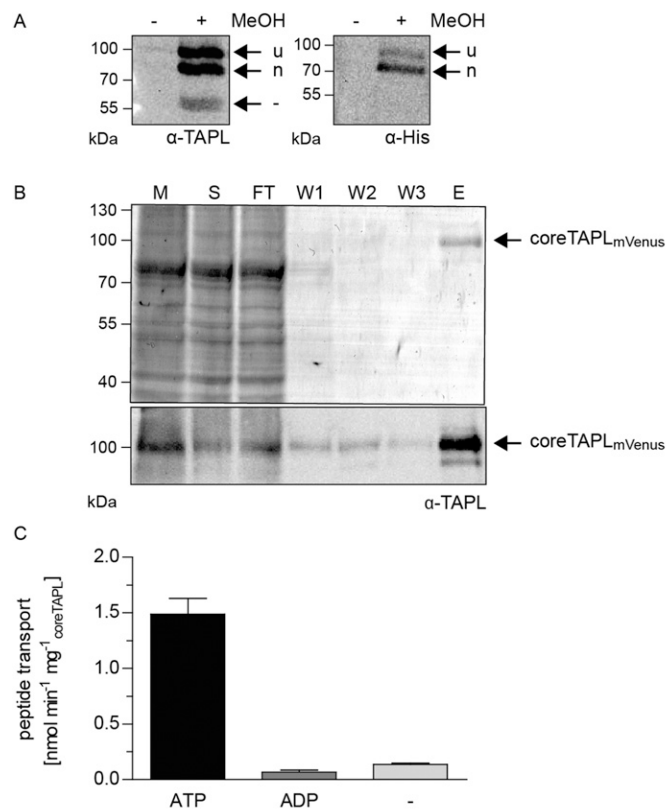

Fig. S2. Expression, purification, and functional reconstitution of coreTAPL$_{mVenus}$. (*A*) Expression of coreTAPL$_{mVenus}$ in *P. pastoris* before and after methanol induction was tested by SDS/PAGE and immunoblotting of crude membranes. Due to incubation for 10 min at 65 °C before loading onto the SDS/PAGE, coreTAPL$_{mVenus}$ is split in two bands depicting native (n) and unfolded (u) mVenus. A band at 55 kDa (–) represents coreTAPL lacking mVenus-His$_{10.}$ (*B*) Coomassie-stained SDS/PAGE and immunoblotting of coreTAPL$_{mVenus}$ purification. CoreTAPL$_{mVenus}$ in crude membranes (M) was solubilized in DDM (S) and purified by metal-affinity chromatography (FT, flow-through; W1-3, wash). Elution (E) was performed with 500 mM imidazole. (*C*) CoreTAPL (2.5 μg) was reconstituted in liposomes (50 μg lipid), composed of a mixture of *E. coli* lipids and DOPC (7:3 mass ratio), and transport was performed for 15 min at 37 °C with 3 μM of RRYC^{ATTO488}KSTEL in the presence of 3 mM Mg-ATP or of Mg-ADP, or in the absence of nucleotide (–). The amount of transported peptide was analyzed by a fluorescence plate reader ($\lambda_{ex/em}$ = 485/520 nm) after denaturation of coreTAPL$_{mVenus}$ for 10 min at 95 °C. The transport assay was performed in triplicate with error bars indicating SD.

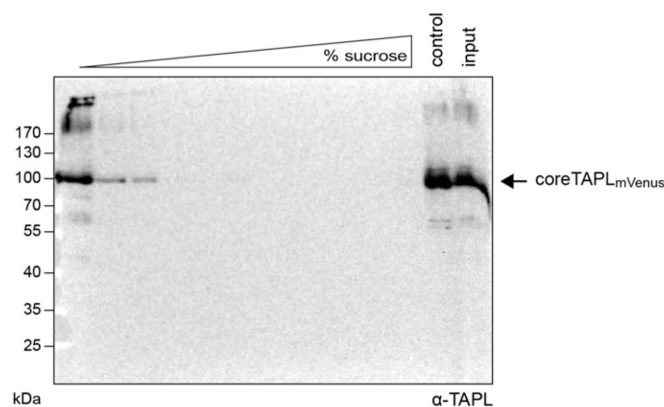

Fig. S3. Reconstitution efficiency of TAPL. Proteoliposomes (1.4 mg lipids) containing coreTAPL$_{mVenus}$ (70 μg) with a protein-to-lipid ratio of 1:20 (wt/wt) were pelleted 30 min at 270,000 × *g*, 4 °C, and resuspended in 100 mM Na_2CO_3, pH 11.5. After 30 min at 4 °C, liposomes were pelleted by centrifugation for 30 min at 270,000 × *g*. The pellet was resuspended in 100 mM Na_2CO_3, pH 11.5, containing 55% (wt/vol) sucrose and overlayed by a sucrose gradient (55–0% sucrose) in 100 mM Na_2CO_3, pH 11.5. After centrifugation for 12 h at 200,000 × *g* and 4 °C, aliquots were analyzed by SDS/PAGE followed by immunoblotting. Then 0.4 μg of purified coreTAPL$_{mVenus}$ (control) was used to calculate the reconstitution efficiency. Untreated proteoliposomes (10 μg lipid) containing coreTAPL$_{mVenus}$ (0.5 μg) served as input control (input); 22% of TAPL was detected in proteoliposomes as quantified by ImageJ 1.46r software.

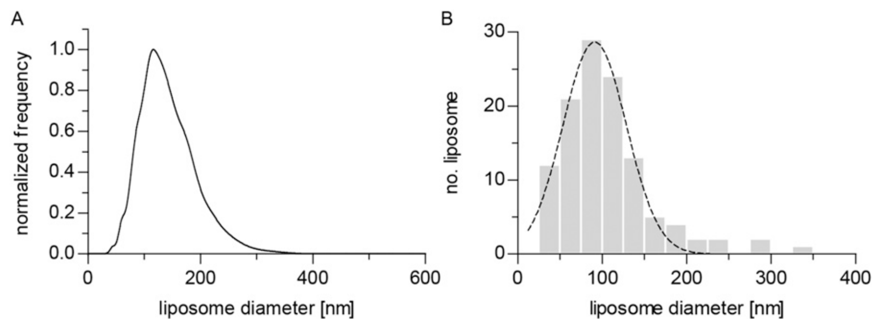

Fig. S4. Size distribution of coreTAPL-containing proteoliposomes. (*A*) By nanoparticle tracking analysis at 25 °C, the mean vesicle size (0.5 µg/mL lipids, n = 3,306) was determined to be 143 ± 49 nm. (*B*) The number of DiD molecules per fluorescence burst was determined using DiD in detergent as standard. The size of the liposomes (n = 115) was calculated, taking the molar ratio of lipid to DiD (4,000:1) and a mean lipid surface area of 0.6 nm^2 into account. A Gaussian fit of the size distribution results in a mean diameter of 91 ± 38 nm.

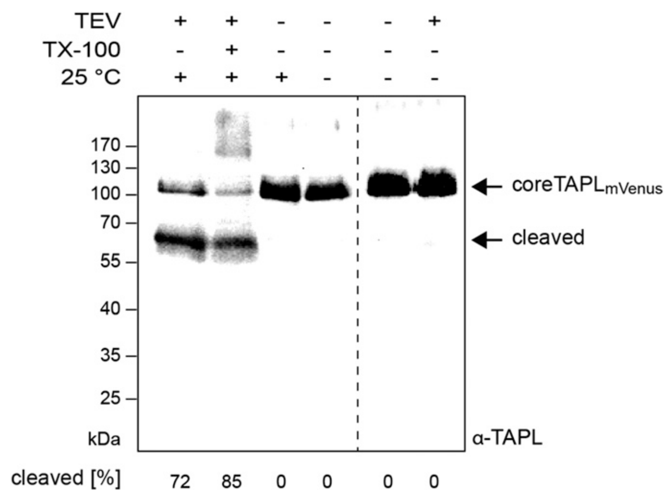

Fig. S5. Orientation of reconstituted coreTAPL$_{mVenus}$. Proteoliposomes (40 µg lipid) containing coreTAPL$_{mVenus}$ (2 µg) were incubated at 25 °C for 1 h in the presence or absence of 1 µg TEV protease. Proteoliposomes solubilized by 1% Triton X-100 indicate the amount of total cleavable protein. Untreated proteoliposomes served as control for quantification by ImageJ 1.46r software.

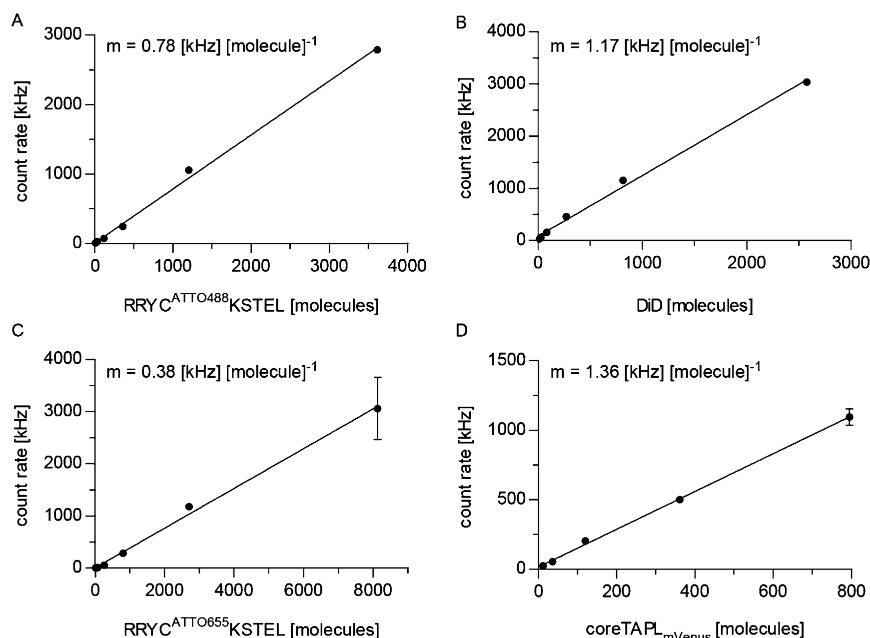

Fig. S6. Quantification of standards for DCFBA. For calibration curves, count rates at the confocal fluorescence microscope were determined for different concentrations of ATTO488- (*A*) or ATTO655-labeled (*C*) RRYCKSTEL peptide in reconstitution buffer with constant laser powers of 488 (3 μW) and 633 (2 μW), or DiD in 10% DDM (*B*), and purified $coreTAPL_{mVenus}$ in Hepes buffer (*D*) with constant laser powers of 488 (4.5 μW) and 633 (1 μW). The respective count rate (kHz) was plotted against the corresponding amount, and the slope (m) of the linear fit is given.

Table S1. Recovery of liposomes

Treatment	Liposome recovery, %	Peptide recovery, %
Centrifuge	100 ± 5	100 ± 8
Filter	18 ± 2	28 ± 7

Transport of 3 μM $RRYC^{ATTO488}KSTEL$ by $coreTAPL_{mVenus}$ (2.5 μg) containing DiD-labeled proteoliposomes was performed for 10 min at 37 °C in the presence or absence of 3 mM Mg-ATP. DiD fluorescence for liposome recovery ($\lambda_{ex/em}$ = 648/670 nm) and ATTO488 fluorescence for recovery of ATP-specific peptide transport ($\lambda_{ex/em}$ = 501/523 nm) were measured in a fluorescence spectrometer after removal of external peptide by either two times centrifugation in 3 mL stop buffer for 20 min at 270,000 × *g* or washing on a 96-well microfilter plate with two times 250 μL of stop buffer (filter). The assays were performed in triplicate with errors representing the SD.