Supporting Information

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SI Text

Materials. Peptides were synthesized as C-terminal amides and purified by RP-HPLC (1); synM2(19-62) was N-terminally acetylated. Details of peptide purification are provided below.

Reagents used in experiments include: POPC, POPG, and DPC (Avanti Polar Lipids); anti-FLAG monoclonal antibody, cholesterol, C14-sulfobetaine, amantadine HCl, and valinomycin (Sigma–Aldrich); rimantadine HCl (TCI America); HPTS, DPX (Invitrogen); and Amberlite XAD-4 (Supelco).

mRNA Synthesis, Culture, and Microinjection of Oocytes. pGEMHJ plasmids encoding WT A/M2 and the A/M2 truncations, with or without C-terminal FLAG tag, were linearized by using the NotI restriction site at the downstream of the gene and in vitro transcription reactions were performed on the linearized DNA by using a T7 mMESSAGE mMACHINE transcription kit (Ambion). X. laevis oocytes were prepared, injected, and maintained as described (2).

Protein Expression in Mammalian Cells. HeLa LTR CD4 βgal cells were maintained in DMEM supplemented with 10% FBS at 37 °C in 5% CO2. Cells grown on cover glass were transfected with pCAGGS constructs by using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol. At 16–20 h posttransfection, the cells were washed and incubated with anti-FLAG monoclonal antibody (Sigma–Aldrich) followed by FITC-conjugated goat anti-mouse antibody (Jackson Immunoresearch Laboratories) in the presence and absence of 0.1% saponin. Labeled cells were fixed in 2% formaldehyde and mounted on a slide. Immunofluorescence images were obtained with a Zeiss LSM 5 Pascal microscope.

Bacterial Expression and Purification of A/M2. C-terminally 6×His-tagged protein was expressed in BL21DE3 Escherichia coli, extracted from inclusion bodies, and purified over a Ni-NTA column, followed by RP-HPLC. For analytical ultracentrifugation, the A/Udorn/72 sequence and a Cys-free W15F variant (W15F, C17S, C19S, C50S) were used for liposome flux measurements; these mutations do not affect proton transport function (3). Details of plasmid preparation are available on request. Detailed expression and purification protocols are provided below.

Protein expression. Two full-length A/M2 constructs were expressed: one sequence corresponded to the WT Udorn strain (GenBank accession no. CAD22815); a second codon-optimized construct had an identical amino acid sequence except all cysteines were mutated to serines (C17S, C19S, C50S), and Trp-15 was mutated to Phe. These changes significantly improve culture yields of protein and do not affect transport function (3). Details of plasmid preparation are available on request. Detailed expression and purification protocols are provided below.

Protein purification. The OG-solubilized supernatant containing A/M2 protein was mixed from BL21(DE3)pLysS E. coli cells (Invitrogen) by using the pET23D(+) plasmid (EMD Biosciences). BL21 cells were chemically transformed with the pET23D(+) plasmid containing the M2 gene and grown in LB media containing 100 μg/mL ampicillin and 50 μg/mL chloramphenicol. Chloramphenicol selection was not used for the Cysless construct. When OD600 of 0.4–1.0 was achieved, protein production was induced by addition of 1 mM IPTG to the media. After 3–5 h of induction, cells were harvested by centrifugation at 4 °C for 30 min. Pellets were resuspended in 25 mL of 50 mM Tris (pH 8), 150 mM NaCl, 40 mM OG, 1 mM lysozyme, 0.02 M PMSF. Solutions were subjected to a minimum of 3 cycles of freezing and thawing in a dry ice/ethanol bath and a 37 °C water bath, followed by another hour at 37 °C for full lysis of cells. This solution was then centrifuged at ~15,000 × g for 30 min and the supernatant was saved for purification on Ni-NTA columns.

Protein purification. The OG-solubilized supernatant containing A/M2 protein was mixed with Ni-NTA agarose (Qiagen) and incubated overnight at 4 °C with constant agitation. Imidazole was added to a final concentration of 10 mM to block nonspecific interactions when purifying the Cys-free construct. Columns were washed successively with 50 mM Tris (pH 8), 150 mM NaCl, 40 mM OG, 1 mM DTT, then 50 mM Tris (pH 8), 20 mM OG, 20% (vol/vol) glycerol, followed by 50 mM Tris (pH 8), 4 mM OG, 20% (vol/vol) glycerol. Finally, the proteins were eluted from the column by using ~15 mL of 50 mM Tris (pH 8), 4 mM OG, 20% (vol/vol) glycerol, and 300 mM imidazole. Fractions were evaluated for protein content by measuring absorbance at 280 nm or by a BCA (Pierce) or Bradford (Sigma–Aldrich) protein assay. Centrifugal concentrators (Millipore) were used for concentrating and rinsing pooled protein fractions into a final solution of 50 mM Tris (pH 8), 4 mM OG, and 20% (vol/vol) glycerol or 50 mM Hepes (pH 8), 4 mM OG, and 20% (vol/vol) glycerol. The protein was then purified by RP-HPLC as described in the next section. Injections onto the HPLC column were made from the final nOg/glycerol buffer above (C17S, C19S, C50S, W15F construct) or from nOg/glycerol buffer diluted by 50% with HPLC buffer B (components described below) (WT construct).

Peptide Purification. Peptides were cleaved from the resin by using cocktails of 90% trifluoroacetic acid, 5% trisopropylsilane, 5% water or, for synM2(19-62), 90% trifluoroacetic acid, 5% thioanisole, 3% ethanedithiol, 2% anisole. After ether or ether/hexanes precipitation, peptides were purified by RP-HPLC on a C4 column out of a trifluoroethanol/aqueous stock buffer with Tris-HCl, pH 8. Peptides were eluted from the column by using linear gradients of buffer A (0.1% trifluoroacetic acid in water) and buffer B (90% acetonitrile, 10% water, 0.1% trifluoroacetic acid) or buffer B’ (60% isopropanol, 30% acetonitrile, 10% water, 0.1% trifluoroacetic acid). Peptides eluted between 50% and 90% of buffer B or B’ depending on hydrophobicity. Lyophilized peptides were then dissolved in organic stock for reconstitution in experiments.

Extinction Coefficients. Protein and fragment concentration was quantified by using absorbance at 280 nm. Calculated extinction coefficient for synM2(22-46) and synM2(22-50) was 5,850 M⁻¹·cm⁻¹. The extinction coefficient used for synM2(19-62) was 6,990 M⁻¹·cm⁻¹; for A/M2 Cys-free, W15F was used for liposome flux assay at 8,480 M⁻¹·cm⁻¹, and for A/M2 it was 13,940 M⁻¹·cm⁻¹.
Liposome Flux Assay. General considerations.

In trials of reconstitution methods for A/M2 and fragments, we found that degree of protein incorporation varied substantially from one procedure to another in a fragment-specific manner. Some conditions led to sample aggregation (often manifest by clogging of the sizing membranes during liposome extrusion). The method described below was found to be effective for all constructs tested, generally yielding reconstitution levels of 70% or more when compared with starting amounts. It was therefore imperative to verify protein incorporation in the final product (e.g., by HPLC as below) instead of relying on the input stoichiometries, because unsuccessful reconstitution methods that we tested often led to very low incorporation (10% or less).

Other determinants of reliable results include: (i) ensuring that the lipids are free of oxidation products (e.g., by using presealed ampules of polar lipids stored as indicated by the manufacturer); (ii) minimizing the time that the protein and lipid are together in a dehydrated state; (iii) confirming that a uniform vesicle population is obtained by using light-scattering and/or electron microscopy; and (iv) minimizing prolonged incubations below neutral pH. When functionally reconstituted, the reaction rate is relatively rapid (4), so we used a rapid mixing device and the assays were conducted at pH 7.4 and 18°C to allow convenient measurement of the signal over 20 s.

The conditions under which the assay is performed necessitate an extremely high degree of proton selectivity in the reconstituted fragment to observe intraliposomal acidification, given the very large excess of K⁺ (inside vesicle) and Na⁺ (in assay buffer) to H⁺. Busath and coworkers (5) have measured a small permeability of the channel to K⁺, but not Na⁺, as determined by a small proton current in the absence of valinomycin. We have also observed a similar valinomycin-independent flux under asymmetric K⁺/Na⁺ conditions, which defines the selectivity ratio of H⁺ versus K⁺ as significantly >10⁶ (at pH 7.4, K⁺ is >10⁶-fold excess over protons under the assay conditions with ~0.1 M K⁺).

Sample preparation. Lipid films (4:1:2 POPC/POPG/cholesterol, 25 µmol total) were made by mixing chloroform stocks of polar lipids and cholesterol in glass vials, then evaporating the chloroform under a stream of argon gas. Films were dried on a lyophillizer before use and stored at −20°C under an argon blanket.

Lyophilized peptides and protein (as trifluoroacetate salts from HPLC purification) were dissolved in ethanol. Stocks were maintained on dry ice or at −80°C until use. Peptide/protein in ethanol in an amount of 25 nmol monomer was added to a lipid film (target 1:1.000 monomer/lipid ratio); additional ethanol was added to a total volume of ~300 µL. For protein-free control liposomes, only ethanol was added. The mixture was vortexed to dissolve the dried lipid, then immediately dried under an argon stream.

The resulting film was immediately hydrated with 995 µL of K buffer (50 mM K₂SO₄, 15 mM KPO₄, pH ~7.5) by vortexing for 2 min. The mixture was frozen on dry ice and thawed. Five microliters of 100 mM HPTS (pyranine; Invitrogen) was then added, and the mixture was vortexed. Unilamellar liposomes were formed by 10 freeze-thaw cycles (dry ice/ethanol and 37°C water bath) and sized by repeated passage through 100-nm polycarbonate filter membranes (Whatman) in a miniextruder (Avestin). Liposomes were then dialyzed for 8 h overnight in a 10K MWCO Pierce Slide-A-Lyzer cassette against 1.8 L of K buffer pH 7.40 (adjusted with H₃PO₄ or NaOH). Liposome extrusion was confirmed on each condition were performed in triplicate. Two outlying runs from the entire dataset of 75 runs were excluded from analysis.

Proton flux assay. Experiments were performed by using an Aviv ATF-105 spectrofluorometer (Aviv Biomedical) in a 1 × 1-cm Hellma quartz fluorescence cell, thermostated at 18°C. The ratiometric pH indicator HPTS (pyranine, pKₐ = 7.22) was used to determine intraliposomal pH. The ratio of fluorescent signal of the deprotonated form (excitation 417 nm, emission 515 nm) to the equilibrium binding.

Assay buffers. Assay buffer [2.5 mL; 50 mM Na₂SO₄, 15 mM NaPO₄ (pH 7.40) adjusted with H₃PO₄ or NaOH] was added to the fluorescence cuvette. Added next was 37.5 µL of 1 M DPX (Invitrogen), a membrane-impermeable quencher of pyranine fluorescence to restrict measured signal to intraliposomal dye. For experiments shown in Figs. 4 and S3, 3.5 µL of 18 µM valinomycin (Fluka/Sigma–Aldrich) in ethanol and 25 µL of 100× aqueous amantadine hydrochloride (Sigma–Aldrich) in a 100:1 (vol/vol) ratio and allowed to interact overnight so as to achieve equilibrium binding.

Liposome flux assay data analysis.

The measured isosbestic kinetic signal (excitation 417 nm, emission 515 nm) was averaged to a single value, and each data point from the deprotonated HPTS signal kinetic (excitation 460 nm, emission 515 nm) was divided by the
isobestic signal average to obtain a signal ratio (deprotonated/isobestic) as a function of time. The ratios at each time point were averaged between the 3 independent experiments run for each condition.

The averaged signal ratio vs. time was converted to intraliposomal pH vs. time based on the calibration curve obtained with free dye in K buffer, as shown in Fig. S4.

Intraliposomal pH (−log[H⁺ free]) vs. time was converted to total intraliposomal [H⁺] vs. time (including H⁺ bound to buffer) by using an estimate of the internal buffering capacity (6) assuming an intraliposomal [phosphate] of 15 mM, and negligible contribution to buffering capacity by the dye given its much lower concentration.

Total intraliposomal [H⁺] vs. time was converted to the total intraliposomal proton count vs. time through multiplication by Nₐ and intraliposomal volume estimated from dynamic light scattering measurements (described below) assuming no phospholipid loss during preparation and a single phospholipid surface area of 6.3 × 10⁻¹⁹ m². Experiments with tracer amounts of radiolabeled lipid show that signal loss during typical preparations is <10%.

The initial number of total protons at t = 3 s was subtracted from successive measurements, and the result was divided by the number of peptide tetramers delivered per experiment, as estimated by integration of the 280-nm protein peak from chromatography of proteoliposome samples on an analytical RP-HPLC column (see below).

Results for protein-free control liposomes shown in Fig. 4 were adjusted for differences in surface area with protein- or fragment-containing liposomes and were normalized for the degree of protein reconstitution observed for the fragment in each panel (i.e., the difference total H⁺ vs. time traces for the control liposomes were divided by the same number of peptide tetramers as determined for the corresponding protein-containing sample shown in each panel).

**Estimate of protein reconstitution.** Liposome samples were mixed in a 2:1 ratio with a lysis buffer containing 150 mM nOG and 150 mM Tris (pH 8); 100 mM TCEP HCl (~6% vol/vol) was added to all samples. The mixture was then injected onto an analytical C4 RP-HPLC column and eluted with a linear gradient of 2:1 isopropanol/acetonitrile with 0.1% TFA and water with 0.1% TFA. Reconstitution was estimated by integration of the 280-nm protein peak in the chromatogram compared with a standard derived from loading synM2(22-46) peptide onto the same column in known amounts.

Using the peptide incorporation method described, reconstitution was nearly complete for synM2(22-46), synM2(22-50), and A/M2 and was ~20% reduced for synM2(19-62).

**Determination of liposome volume and surface area.** Liposome radii were determined by using a Wyatt DynaPro dynamic light scattering instrument at 25 °C. Liposomes were diluted ~1:1,000–1:10,000 into prefiltered K buffer. Measurements were performed in triplicate for each sample, and the radius distribution peak maxima were averaged.

Volumes and surface areas were determined based on the averaged measured radius. The software of the instrument was set for analysis based on a Rayleigh sphere model, and the solvent model was set to PBS.

**AUC. Sample Preparation.** AUC samples were prepared from ethanol stock solutions of the A/M2 protein and synM2(22-46) peptide. The ethanol was removed from the sample aliquots by high vacuum centrifugation followed by lyophilization. Detergent containing sample buffer was used to solubilize the dried protein films. Sample buffers were supplemented with ³H₂O such that the buffer density was equal to the density of the detergent solution (7–9). The sample solution for WT A/M2 (~50 μM) contained 60 mM Tris-HCl/Na₃C₆H₅O₇/Na₃PO₄ (1:1:1) buffer containing 100 mM NaCl, 2 mM TCEP, 7.5 mM C₁₄-sulfobetaine, and ~17.7% ³H₂O. The sample solution for synM2(22-46) (~60 or 100 μM) contained 50 mM Tris·HCl, 2 mM TCEP, 100 mM NaCl, 15 mM DPC, and ~52.5% ³H₂O. For amantadine binding studies, a 3- to 10-fold molar excess of amantadine HCl (depending on detergent conditions) was added to the buffer solutions. The pH was adjusted by using HCl and NaOH to within (+/− 0.05 pH units) before density determination and was not corrected for ³H₂O concentration. The pH was measured before and after centrifugation experiments to confirm sufficient buffering.

**AUC data collection.** Absorbance scans from 250–320 nm were collected for the samples at 3,000 rpm to determine the precise concentration. The extinction coefficients at 280 nm were adjusted for the experimental buffer as determined (10). The protein monomer molecular mass and partial specific volume, corrected for partial deuteration, were calculated by using the program SEDENTRP (11) modified to include updated amino acid specific volumes (12). Data were collected when samples reached equilibrium at 3–4 speed intervals ranging from 15,000 to 40,000 rpm based on average complex molecular weight as recommended (11, 13).

**AUC data analysis.** A single-species model was first fit to radial absorption profiles for the sample to determine the apparent molecular weight. To rule out other populated aggregation states, the data were also analyzed according to various monomer-oligomer schemes (7) by globally fitting to several equilibria theoretical curve sets using Igor Pro (Wavemetrics). Only a fully cooperative monomer-tetramer equilibrium gave a satisfactory fit to the data.

The pH dependence of M2 tetramerization involves multiple histidine protonation states in the monomeric and tetrameric forms of the protein. The observed equilibrium dissociation constant for tetramerization K—isobestic for the tetramer observed at high pH by:

\[
K_{obs} = \frac{[\text{mon}] + [\text{mon} \cdot H^+]}{[\text{tet}] + [\text{tet} \cdot H^+]} = \frac{1}{K_{tet} \left( 1 + \frac{[H^+]}{K_{mon}} \right)}
\]

in which Kₘon is the equilibrium constant for protonation of the monomer, while K[1] through K[4] are for protonation of His-37 in the tetramer. To allow the use of published acid dissociation constants (14), these were treated as ensemble averages as in previous work and were not corrected for statistical factors associated with the number of permutations of protonation states for a given charge.
state. Previously determined values of $K[1]$ through $K[4]$ were used in fitting curves to the data, leaving only the value of $K_{tet}$ for synM2(22-46) or $K_{tet}$ and $K_{mon}$ as adjustable parameters.

**CD.** Experiments were run with $\approx 50 \mu M$ absolute concentration of monomeric peptide. Peptide films were taken up in 50 mM Tris (pH 8.0) with 5 mM DPC for synM2(22-46) S31N, and in 10 mM Phosphate, pH 7.4 with 2.5 mM DPC for synM2(22-46) WT. The resulting solution was vortexed, then freeze/thawed and briefly sonicated to ensure complete solubilization. Final peptide monomer/detergent ratios were 1:41 (WT) and 1:50 (S31N). Concentrations of reconstituted peptides were verified by UV-Vis spectroscopy. Aqueous stocks of rimantadine and amantadine HCl were lyophilized; the resulting solids were taken up in peptide reconstitution buffer with the detergent component omitted. Inhibitor concentration in these stocks was 400 $\mu M$.

**ITC.** Protein/lipid films were hydrated in 50 mM potassium sulfate, 15 mM potassium phosphate, pH 7.4, vortexed for 2 min and sonicated in a bath sonicator (G112SP1G; Laboratory Supplies) to create small unilamellar vesicles (SUVs).

ITC was conducted by using a high-precision VP-ITC titration calorimetric system (MicroCal). The calorimetric cell ($\approx 1.4 mL$), which contained either reconstituted synM2(22-46) (10 $\mu M$ total peptide monomer concentration) or lipid vesicles alone, was titrated with 100 $\mu M$ amantadine in the same buffer in steps of 10-$\mu L$ injections at 25 °C. All solutions were properly degassed to avoid formation of bubbles in the calorimeter during stirring.

Detection of the M2(21-51)-FLAG, M2(21-61)-FLAG, and full-length A/M2-FLAG in oocytes (used for relative specific activity measurements) and mammalian cells. (A) Oocytes injected with M2(21-51)-FLAG, M2(21-61)-FLAG, and full-length A/M2-FLAG mRNAs that expressed robust inward currents were fixed with paraformaldehyde. Oocytes were treated for indirect immunofluorescence by anti-FLAG antibody in the presence or absence of 0.1% saponin. Several uninjected oocytes were also measured. (B) To confirm expression, HeLa LTR CD4βgal cells were transfected with M2(21-51)-FLAG, M2(21-61)-FLAG, and the full-length A/M2-FLAG in pCAGGS constructs. At 16–20 h posttransfection, the cells were stained for indirect immunofluorescence by using anti-FLAG antibody in the presence and absence of 0.1% saponin. These findings indicate that WT A/M2-FLAG, M2(21-61)-FLAG, and M2(21-51)-FLAG proteins are expressed and appropriately inserted into the cytoplasmic membrane with the C terminus facing inside the cells.
Fig. S2. Current–voltage relationship of M2(21-51)-FLAG, M2(21-61)-FLAG, and full-length M2-FLAG proteins expressed in oocytes. A voltage ramp from –50 to 60 mV was applied after the oocyte displayed maximum inward current at pH 5.5. Oocyte membranes do not withstand polarization to very positive voltages (>60 mV), because they either become leaky or develop large endogenous currents, therefore insufficient positive voltage was able to be applied to achieve reversal of current for many oocytes. Current–voltage relationship was plotted after subtraction of pH 8.5 background current from pH 5.5 current.
Fig. S3. synM2(22-50) proton fluxes determined by proteoliposome assay with an electrical potential (−120 mV) \( \Delta p \) and \( \Delta p_{\text{out}} \) of 7.40. Cumulative protons per tetramer are plotted vs. time after liposome dilution into assay buffer after a 3-s delay for mixing. Protein-free control liposomes exhibit minimal background flux. The proteoliposome proton flux was also studied at a high concentration of amantadine (99 \( \mu \)M) and was nearly fully inhibited.
Fig. S4. HPTS pH calibration curve and fitting parameters.

\[ y = \frac{2.6893}{1 + \frac{x}{5.3368 \times 10^{-8}}} \]

\[ R = 0.99994 \]