Supplementary Methods

Heterotypic cell-cell fusion assay. QM5 cells were labelled with CMTMR as per the manufacturers instructions (Molecular Probes), then transfected with pcDNA3 expressing p14 as previously described (Corcoran and Duncan, 2004). At 3 h post-transfection, cells were removed from the tissue culture dish with 10 mM EDTA in PBS and seeded on non-transfected CMAC-labeled target cells grown on glass coverslips. Cells were co-cultured for 2 h to allow p14-mediated cell-cell fusion, washed with HBSS, fixed with methanol, mounted on glass slides using fluorescent mounting media (Dako), and visualized and photographed using a Zeiss LSM510 scanning argon laser confocal microscope (see Suppl. Fig. 1).

Syncytial assay: QM5 cells were transfected with pcDNA3 expressing authentic p14 or a p14 construct containing the C-terminal His-tag and enterokinase cleavage site. Cells were fixed with methanol and stained with Wright-Giemsa, and observed by light microscopy for the presence of multinucleated syncytia indicative of p14 membrane fusion activity (see Suppl. Fig. 1).

Construction of recombinant baculovirus expressing p14. A recombinant baculovirus expressing p14 was generated using the Bac-To-Bac Baculovirus Cloning and Expression System, as described by the manufacturer (Invitrogen). Briefly, p14 was cloned into the SalI and NotI sites of pFastBac1 after PCR amplification (Forward primer: 5'-TAGATGTCGACCCACC ATGGGGAGTGGACCCTCTAATTTCGTC-3'; Reverse primer: 5'-TGAAGAAGCGGCCGC GTTAGTGATGGTGGTGATGGTGCTTGTCGTCGTCATCAATGGCTGAGACATTATCGA TGTTGACGTATGT-3'), incorporating an in-frame enterokinase-cleavable, C-terminal 6x His tag. The plasmid sequence was confirmed and transformed into competent bacmid-containing DH10BAC *E.coli* cells (Invitrogen). Recombinant bacmid DNA was isolated and used to transfect SF21 cells (4×10^6) using CellFECTIN in SF900II medium for 5 h at 27 °C. The

transfection medium was replaced with SF900II supplemented with 3% fetal calf serum. After a 3-day incubation at 27 °C, the recombinant baculovirus-containing supernatant was harvested and passaged three times in SF21 cells using an MOI of 0.01 – 0.1. The recombinant baculovirus titer was obtained by plaque assay.

Purification of p14. SF21 cells were grown in 1.5 L suspension cultures to a density of 4 x 10^6 cells/ml, infected with p14-expressing baculovirus at an MOI of 0.05 – 0.08, and shaken at 27^{o}C and 135 RPM for 48 h The frozen cell pellets were lysed with extraction buffer (50 mM sodium phosphate, 300 mM NaCl, 1.6 % Igepal, pH 7.0) plus protease inhibitors (200nM aprotinin, 1 μM leupeptin, and 1 μM pepstatin (Sigma)). The lysate was pelleted and the supernatant added to pre-equilibrated Talon metal affinity resin (Clontech). The resin was rocked at 4^{o}C overnight, washed twice with extraction buffer, and eluted using elution buffer (50 mM sodium phosphate, 300 mM NaCl, 150 mM imidazole, 1.6 % Igepal, pH 7.0). The eluate was dialyzed three times for 40 min at 4^{o}C against 50mM HEPES, 150 mM NaCl, 1.6% Igepal, pH 6.8, centrifuged at 15,000 x *g* for 25 min, and the supernatant loaded onto a 5ml volume HiTrap SP HP ion exchange column (Amersham Pharmacia Biotech). The column was washed with five column volumes of Buffer 1 (50 mM HEPES, 185 mM NaCl, 1.6% Igepal, pH 6.8), five column volumes of Buffer 2 (50 mM HEPES, 1 M NaCl, 1.6% Igepal, pH 6.8), and then ten column volumes of Buffer 1. The supernatant from the cell lysate was loaded onto the column at a rate of 5ml/min. The column was washed with two column volumes of Buffer 3 (50 mM HEPES, 185 mM NaCl, 1.6% Igepal, pH6.8) followed by two column volumes of Buffer 4 (50mM HEPES, 185 mM NaCl, 1.6% OG, pH 6.8), and the bound p14 eluted using ten 0.5ml aliquots of Buffer 5 (50 mM HEPES, 300 mM NaCl, 1.6% OG, pH 7.7). The purity of the protein was determined following analysis by SDS-PAGE and silver-staining, and was estimated to be >95%. Protein concentration

(1.5-2 mg/ml) was estimated using a Lowry assay with BSA as standards. Samples of the various purification stages are shown in Suppl. Fig. 2A. The purified protein contained the essential N-terminal myristate moiety as shown by radiolabeling with [^{3}H]myristic acid (Suppl. Fig. 2B).

Atomic force microscopy measurements: Atomic force microscopy (AFM) was used to examine the adhesive property of p14-liposomes. AFM is based on the detection of repulsive and attractive surface forces between a probe tip attached to a flexible cantilever and the surface of the sample being scanned (Kumar and Hoh, 2001). In the linear regions of the force curves the tip is in contact with the surface. On further retraction of the sample, the cantilever eventually detaches from the surface and assumes its neutral position. The transition region gives information about the nature of the interaction between the tip and the sample surface, the extent of which is proportional to the interaction force (Santos and Castanho, 2004).

Atomic force microscopy (AFM) force measurements were recorded at room temperature using a Molecular Imaging Microscope. V-shaped cantilevers with oxide sharpened Si_3N_4 tips were used with spring constants 0.01N/m. All force measurements between clean AFM tips and lipid membranes, lipid membranes with protein p14, or p14 alone adsorbed on fresh cleaved mica surfaces were performed in Hepes buffer. The retraction force curves were recorded at a rate of 0.5µm.s^{-1} (see Suppl. Fig. 3).

Membrane fusion and endocytosis inhibitor studies: All lysosphotidylcholine (LPC) and endocytosis inhibitor studies were carried out as described in the lipid-mixing assay, with the following exceptions. *LPC:* Proteoliposome suspensions and cells were incubated with the indicated concentration of LPC for 5 min at 37^{o}C and then chilled to 4^{o}C for 30 min before addition of proteoliposomes to cells which were incubated at 37ºC in the presence of LPC. This treatment inhibited p14-mediated liposome-cell fusion (Suppl. Fig. 4A), but did not cause

liposome lysis as measured by optical density (600nm). *Endocytosis inhibitors:* The inhibitor cocktail consisted of dansylcadaverine (50μM), monensin (25 μM) and nystatin (2.5 μM) (inhibitors of clatherin-mediated endocytosis, endosomal acidification and calveolae-mediated endocytosis, respectively) (Decorti et al., 1999). The endocytosis inhibitors inhibited the endocytic entry of reovirus by ~90%, as determined previously using a plaque-reduction assay (Duncan, 1996). The inhibitor cocktail was incubated with cells for 1 h at 37^{o}C. The cells were washed with HBSS twice and chilled in HBSS containing the same amounts of inhibitors, after which inhibitor-supplemented liposome suspensions were added and the lipid-mixing assay conducted as described above, using a 20 min incubation period at 37^{o}C (see Suppl. Fig. 4B).

Western blotting: The topology of p14 in the membrane of liposomes was determined using an anti-peptide antiserum raised against the p14 N-terminal ectodomain and a polyclonal antibody specific for the C-terminal enterokinase tag. To confirm that both antibodies were capable of recognizing p14, serial five-fold dilutions of purified p14 were resolved by SDS-PAGE and blotted onto PVDF membranes. The membranes were blocked in blocking buffer (Tris-buffered saline (TBS) with 0.01% Tween 20 (TBST) plus 4% skimmed milk powder) at 4^{o}C overnight, then probed with each primary antiserum (1/10,000) diluted in blocking buffer for 1 h at room temperature. Blots were washed extensively with TBST, then probed with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (1/10,000) diluted in blocking buffer for 1 h at room temperature. Blots were washed extensively with TBST, then developed using the LumiGLO Chemiluminescent Substrate System (KPL, MD) according to the manufacturer's instructions. The dried membrane was exposed to X-ray film (Hyperfilm ECL, Amersham Biosciences) for periods of 30 sec to 3 min (see Suppl. Fig. 5).

p14 surface-density estimations. The p14 concentration in liposomes was quantified by SDS-PAGE and silver staining, using serial protein dilutions of purified p14 whose protein concentration was estimated using a Lowry assay with BSA as a standard. The molecular weight of p14 (15886.4 with the myristic acid modification) was used to determine moles of p14 per liposome. A lipid-phosphorus assay (Rouser, 1966) was used to quantify the concentration of phospholipids in the liposome preparations. The protein:lipid molar ratios were calculated, using the estimate of 1.657×10^6 phospholipids per 400 nm liposome (Cullis et al., 1996). To estimate the surface density of p14 per μm^2, liposomes were assumed to be spherical and uniformly 400 nm in diameter, and only phospholipids were assumed to contribute to the surface area of liposomes.

Supplementary References

Corcoran, J.A. and Duncan, R. (2004) Reptilian reovirus utilizes a small type III protein with an external myristylated amino terminus to mediate cell-cell fusion. *J Virol*, **78**, 4342-4351.

Cullis, P.R., Fenske, D.B. and Hope, M.J. (1996) Physical properties and functional roles of lipids in membranes. In Vance, D.E. and Vance, J. (eds.), *Biochemistry of lipids, lipoproteins and membranes*. Elsevier, Amsterdam, Vol. 31, pp. 1-33.

Decorti, G., Malusa, N., Furlan, G., Candussio, L. and Klugmann, F.B. (1999) Endocytosis of gentamicin in a proximal tubular renal cell line. *Life Sci*, **65**, 1115-1124.

Duncan, R. (1996) The low pH-dependent entry of avian reovirus is accompanied by two specific cleavages of the major outer capsid protein mu 2C. *Virology*, **219**, 179-189.

Kumar, S. and Hoh, J.H. (2001) Probing the machinery of intracellular trafficking with the atomic force microscope. *Traffic*, **2**, 746-756.

Rouser, G., Siakotos, A.N., and Fleisher, S. (1966) Quantitative analysis of phospholipids by thin -layer chromatography and phosphorus analysis of spots. *Lipids*, **1**, 85-86.

Santos, N.C. and Castanho, M.A. (2004) An overview of the biophysical applications of atomic force microscopy. *Biophys Chem*, **107**, 133-149.