Interaction(s) of rotavirus non-structural protein 4 (NSP4) C-terminal peptides with model membranes

Huan HUANG*, Friedhelm SCHROEDER*, Mary K. ESTES†, Tanya McPHERSON‡ and Judith M. BALL‡¹

*Department of Pharmacology and Physiology, Texas A&M University, TVMC, College Station, TX 77843-4466, U.S.A., †Division of Molecular Virology and Microbiology, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030, U.S.A., and ‡Department of Pathobiology, Texas A&M University, TVMC, College Station, TX 77843-4467, U.S.A.

Rotavirus is the major cause of dehydrating gastroenteritis in children and young animals. NSP4 (non-structural protein 4), a rotaviral non-structural glycoprotein and a peptide NSP4_{114–135} (DKLTREIEQVELLKRIYDKLT), corresponding to NSP4 amino acids 114–135, induce diarrhoeal disease in a neonatal mouse model and interact with model membranes that mimic caveolae. Correlation of the mechanisms of diarrhoea induction and membrane interactions by NSP4 protein and peptide remain unclear. Several additional NSP4 peptides were synthesized and their interactions with membranes studied by (i) CD, (ii) a filtration-binding assay and (iii) a fluorescent molecule leakage assay. Model membranes that varied in lipid compositions and radius of curvature were utilized to determine the compositional and structural requirements for optimal interaction with the peptides of NSP4. Similar to the intact protein and NSP4_{114–135}, peptides overlapping residues 114–135 had significantly higher affinities to membranes rich in negatively charged lipids, rich in cholesterol and with a high radius of curvature.

In the leakage assay, small and large unilamellar vesicles loaded with the fluorophore/quencher pair 8-aminonaphthalene-1,3,6-trisulphonic acid disodium salt/*p*-xylene-bis-pyridinium bromide were incubated with the NSP4 peptides and monitored for membrane disruption by lipid reorganization or by pore formation. At a peptide concentration of 15 μ M, none of the NSP4 peptides caused leakage. These results confirm that NSP4 interacts with caveolae-like membranes and the α -helical region of NSP4_{114–135} comprises a membrane interaction domain that does not induce membrane disruption at physiological concentrations.

Key words: CD, enterotoxin, fluorescence, liposome leakage, peptide–membrane interaction, rotavirus non-structural protein 4 (NSP4).

INTRODUCTION

Rotaviruses are major pathogens causing severe, life-threatening dehydrating gastroenteritis in children and animals worldwide, and in immune-compromised and elderly adults [1–3]. Rotavirus NSP4 (non-structural protein 4), a transmembrane ER (endoplasmic reticulum)-specific glycoprotein, was found to play important roles in rotavirus morphogenesis, and to function as the first known viral enterotoxin [4]. Previous studies of NSP4 revealed the presence of three hydrophobic regions close to the N-terminus, a single transmembrane domain, two glycosylation sites within the short ER lumenal domain and an extended cytoplasmic tail [5]. Crystallographic analysis of residues 95–137 revealed an α -helical, tetrameric coiled-coil structure [6]. The helical structure of each monomer is similar with an approximate length of 58 Å (1 Å = 0.1 nm) and a highly charged surface in which the charged residues follow the curvature of the coiled-coil. Furthermore, there is a bivalent cation-binding site, probably calcium, located approx. four helical turns from the base of the coiled-coil, which associates with a network of water molecules and enhances the stability of the homotetramer [6].

Purified NSP4 and a peptide NSP4_{114–135} (DKLTREIEQVELLKRIYDKLT), corresponding to NSP4 residues 114–135, induce diarrhoea in young (6–10-day old), but not adult, CD1 mice, akin to the natural infection. Diarrhoea induction occurs in the absence of histological alterations, it is age- and dose-dependent and specific [4]. Increasing evidence indicates that NSP4 func-

tions by activating a signal transduction pathway, which increases [Ca²⁺]_i (intracellular calcium) levels by mobilizing calcium from the ER [7,8], resulting in chloride secretion [4,9]. This signalling event necessitates the transport of NSP4 from the ER to the plasma membrane for release from the cell to interact with surface-signalling molecules. Classical ER retention signals are lacking in the NSP4 sequence and the mechanism of NSP4 N-terminal retention in the ER is unknown. However, NSP4 does not appear to be retrieved from the Golgi by retrograde transport since NSP4 carbohydrate moieties remain sensitive to endoglycosidase H digestion (oligosaccharide processing stops at Man₈GlcNAc) [5,11]. To determine if components of the ER quality control system contribute to NSP4 ER retention, host chaperone proteins, immunoglobulin heavy-chain binding protein (GRP78) and endoplasmic reticulum (GRP94), that reside in the ER lumen, have been examined and found to be up-regulated in response to NSP4 expression in mammalian cells, but fail to associate directly with NSP4 [12]. In contrast, calnexin, a chaperone *trans*-ER type I membrane protein that interacts with assembly intermediates of numerous membrane glycoproteins, associates with glycosylated NSP4 *in vivo* and *in vitro* [13]. Calnexin interacts with trimmed NSP4 during a viral infection even though glucose trimming is not required for assembly of infectious virions. Although the precise intracellular transport pathway is unknown, a recent study shows that NSP4 undergoes a distinct proteolytic cleavage that releases a C-terminal fragment, NSP4_{112–175}, which is transported to the cell surface and released into culture media via a

Abbreviations used: ANTS, 8-aminonaphthalene-1,3,6-trisulphonic acid disodium salt; CFTR, cystic fibrosis transmembrane conductance regulator; DOPS, 1,2-dioleoyl-*sn*-glycero-3-[phospho-L-serine]; DPX, *p*-xylene-bis-pyridinium bromide; ER, endoplasmic reticulum; LUV, large unilamellar vesicles; NSP4, non-structural protein 4; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; PS, phosphatidylserine; SUV, small unilamellar vesicles; TFE, trifluoroethanol; TNBS, 2,4,6-trinitrobenzenesulphonic acid.

¹ To whom correspondence should be addressed (e-mail jball@cvm.tamu.edu).

microtubule-dependent pathway [14]. Alternatively, the increase in cytoplasmic calcium concentration could be explained by membrane destabilization activity that causes calcium release from the ER [4]. A separate study showed that amino acids 48–91 of NSP4 possess membrane-destabilizing activity, whereas NSP4_{114–135} by itself had no effect on membrane stability. However, the membrane-destabilizing activity of peptide 48–91 was potentiated when NSP4_{114–135} was also present [15].

In addition to its enterotoxic activity, NSP4 plays an important role in the rotavirus maturation process by functioning as an intracellular receptor at the ER [16,17]. Subviral particles bind NSP4 C-terminal residues [18,19] in a unique morphogenetic process whereby the immature double-layered particles bud into the ER and obtain the outer capsid protein, a spike protein and a transient membrane envelope [20]. Maturation of infectious particles takes place in the ER lumen, where the ER membrane is removed by an unknown mechanism [20]. NSP4 and NSP4_{114–135} are postulated to play a role in disruption of this transient envelope but the mechanism remains unclear [21]. It has also been shown that during the final stage of viral maturation, NSP4 is associated with rafts, a membrane microdomain rich in sphingolipids and cholesterol, which provide the platform for virus assembly [22].

Membrane interactions might play an important role in the biological functions of NSP4 and its active peptides. Understanding these membrane interaction properties is probably essential to determine NSP4 ER retention, role in formation and removal of the transient membrane envelope during viral assembly, membrane-destabilizing activities, association with rafts and targeting to and interaction with membrane domains rich in proteins involved in calcium signalling.

In the present study, the interaction with model membranes of several peptides related to the active enterotoxic NSP4_{114–135} was studied by CD and a filtration-partitioning assay, and membrane-destabilization activity was studied by a leakage/quenching fluorescent assay. These experiments were designed to address the following questions: (i) Do these peptides bind to lipid membrane vesicles? (ii) How do the membrane interactions affect the secondary structures of the peptides? (iii) How do the structures of the peptides correlate with the membrane interaction activities? (iv) What are the compositional and structural requirements of the lipid vesicles for optimal interaction with these peptides? (v) Do the membrane interactions of the peptides cause membrane disruption or fusion? (vi) Do the membrane interactions correlate with the enterotoxic activities?

EXPERIMENTAL

Materials

Phospholipids DOPS (1,2-dioleoyl-*sn*-glycero-3-[phospho-L-serine]) and POPC (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine) were obtained from Avanti Polar Lipids (Alabaster, AL, U.S.A.). Cholesterol, *o*-phthalaldehyde, TFE (trifluoroethanol), 2-mercaptoethanol, melittin (approx. 93% HPLC), Sephadex G-50 for gel filtration and Pipes were purchased from Sigma (St. Louis, MO, U.S.A.). Microcon-100 filtration units were obtained from Fisher Scientific (Pittsburgh, PA, U.S.A.). ANTS (8-aminonaphthalene-1,3,6-trisulphonic acid, disodium salt) and DPX (*p*-xylene-bis-pyridinium bromide) were purchased from Molecular Probes (Eugene, OR, U.S.A.). Synthetic peptides were purchased from the University of Pittsburgh Peptide Synthesis Core (Pittsburgh, PA, U.S.A.) or were synthesized by Texas A&M University Peptide Core. The peptides correspond to the SA11 rotavirus NSP4 sequence [11].

Diarrhoea induction

To correlate structure with function, the NSP4 synthetic peptides were tested for diarrhoea induction in our young pup model as described in [4]. Briefly, 6–10-day-old pups were given 50–100 nmol of peptide by intraperitoneal injection. The pups were then monitored for diarrhoea from 4 to 12 h post injection and then again at 24 h post injection. The severity of diarrhoea was scored from 1 to 4+ as described in [4].

Preparation of SUV (small unilamellar vesicles)

SUV comprising different percentages of the neutral lipid POPC, cholesterol and the negatively charged lipid DOPS were prepared by sonication using a modification of a method described previously [23]. The lipids in chloroform stock solutions were measured and mixed in an amber glass vial. The solvents were removed under N₂ with constant rotation so that the dried lipids formed a thin film on the glass wall. Mops buffer (10 mM, pH 7.4, prefiltered through a 0.2 µm filter; Millipore, Bedford, MA, U.S.A.) was then added; the sample was vortex-mixed, bath-sonicated and vortex-mixed again. The resultant multilamellar membrane suspension was sonicated with a microprobe under N₂ at 4 °C. Sonication was performed with a Sonic Dismembrator Model 550 (Fisher Scientific) at energy level 3.5 using 1 min pauses after every 2 min sonication to prevent overheating of the lipid solution. The sonicated solution was centrifuged using a 40Ti rotor (Beckman, Fullerton, CA, U.S.A.) at 110 000 *g* for 4 h to remove any multilamellar vesicles and any titanium debris from the sonicator probe. The lipid concentration of the final SUV solution was determined by phosphate assay as described previously [24].

Preparation of LUV (large unilamellar vesicles)

Extrusion through polycarbonate membranes was used to prepare LUV comprising a known amount of POPC, cholesterol and DOPS. All procedures were the same as described above for preparation of SUV, except that the sonication step was omitted. Instead, the lipid suspension was freeze-thawed five times, and then extruded 21 times (back and forth) through double layers of 0.1 µm polycarbonate membranes with a mini-extruder (Avanti Polar Lipids).

CD and secondary-structure estimation

The interactions of NSP4_{114–135}, NSP4_{131K} (DKLTTREIEQV-ELLKRIKDKLT), D-NSP4_{114–135} (identical in sequence with the 114–135 peptide, except that D-amino acids were used for the synthesis) and NSP4_{120–147} (EIEQVELLKRIYDKLTVQTTG-EIDMTKE) peptides with SUV and LUV were determined by CD. The CD spectra were obtained with the following modifications of a previously reported procedure [23]. The samples contained 15 µM peptide with or without lipid vesicles (1 mM) in 10 mM phosphate buffer (pH 7.4, prefiltered through 0.2 µm filter). Samples comprising buffer or lipid vesicle without peptide were used for background correction. CD spectra were obtained at room temperature (25 °C) in a 1 mm circular quartz cell and a Model J-710 Spectropolarimeter (JASCO, Easton, MD, U.S.A.). Each CD spectrum was recorded from 185 to 260 nm with step resolution of 1 nm, speed of 50 nm/min, response of 1 s, bandwidth of 2.0 nm and sensitivity of 10 mdeg. For each determination, 5–10 scans were averaged, smoothed, background-subtracted and converted into mean residue molar ellipticity [θ] (deg · cm²/dmol).

The percentage helix for the NSP4 peptides was estimated using the equation $\theta_{222} = (f_h - i\kappa/N)[\theta_{h\ 222\infty}]$ [25,26], where θ_{222} is the mean residue θ value at 222 nm, f_h is the fraction in α -helical form, i is the number of helices, κ is a wavelength-specific constant with a value of 2.6 at 222 nm, N is the number of residues in the peptide and $\theta_{h\ 222\infty}$ is the molar ellipticity for a helix of infinite length at 222 nm (i.e. $-39\ 500\ \text{deg} \cdot \text{cm}^2/\text{dmol}$) [25,26].

Filtration partitioning assay

The interaction of NSP4 peptides with membranes was also determined by a filtration assay [27]. A reaction mixture of 4 nmol of NSP4 peptide and 267 nmol of lipid vesicles in a total volume of 200 μl of 10 mM Mops buffer (pH 7.4) was incubated at room temperature for 10 min. Each sample was then passed through a filter with a molecular-mass cut-off of 100 kDa by centrifugation at 3000 g for 4–7 min using a Microcon-100 filtration unit, until only 5–10 μl remained in the filter unit. Each filter was rinsed with 100 μl of buffer and centrifuged again for 2–3 min. Unbound peptide in the flow-through fraction was quantified by an *o*-phthalaldehyde assay and compared with standard curves comprising 1–6 nmol of NSP4 peptides. In the *o*-phthalaldehyde assay, 0.5 ml of 0.05 M sodium borate buffer (pH 10) and 0.5 ml of 0.5 % (v/v) 2-mercaptoethanol in ethanol were added to 300 μl aliquots of the NSP4 peptides in the flow through. The samples were vigorously vortexed after each addition. A stock solution of *o*-phthalaldehyde (10 mg/ml in methanol) was diluted 10-fold in 0.05 M borate (pH 10) and a 0.5 ml aliquot of this diluted stock solution was added to the samples while they were vortexed. The mixture was incubated at room temperature for 20 min. Fluorescence intensity ($\lambda_{\text{ex}} = 340\ \text{nm}$; $\lambda_{\text{em}} = 440\ \text{nm}$) was determined with a PC1 Photon Counting Fluorescence Spectrophotometer (ISS, Champaign, IL, U.S.A.). The fluorescence signal is directly proportional to NSP4 peptide concentration over the concentration range used in this study.

Photon correlation spectroscopy to determine the size of SUV and LUV in the presence and absence of NSP4_{114–135}

The particle size distribution of SUV and LUV with and without NSP4_{114–135} was performed using a Beckman Coulter (Miami, FL, U.S.A.) using a Coulter N4 Plus Photon Correlation Spectrometer, which is suitable to measure particles 0.003–3 μm in diameter. This method gave the mean size and size distribution for each sample.

Membrane transbilayer distribution of PS (phosphatidylserine)

The membrane transbilayer distribution of PS was determined with TNBS (2,4,6-trinitrobenzenesulphonic acid), a non-penetrating chemical probe, as described previously [28].

Liposome leakage assay

First, SUV and LUV containing the fluorophore/quencher pair (ANTS/DPX) were prepared. Stock solutions of POPC, cholesterol and DOPS in chloroform were measured, mixed, evaporated to dryness under N_2 , and dried under vacuum for at least 4 h. The dried lipids were hydrated with dye-containing Pipes buffer (5 mM ANTS/50 mM DPX/20 mM Pipes/27.5 mM NaCl, pH 7.4) to yield a 50 mM lipid suspension. The suspension was vortexed for 1 min, bath sonicated for 5 min and vortexed again for 1 min. This suspension was subjected to probe sonication and then ultracentrifuged to make SUVs, or subjected to

15 freeze–thaws and extrusion through two layers of polycarbonate membrane (pore size 0.1 μm) to prepare LUVs as described above. The ANTS/DPX-containing vesicles were separated from free dye by size-exclusion chromatography using Sephadex G-50. The fractions containing a high concentration of vesicles and no free dye were pooled, and the lipid concentration was determined by the phosphorus assay as described above.

Secondly, the induction of vesicle leakage was monitored by the fluorescence intensity of the released fluorescent dye ANTS, which is quenched by the DPX quencher within the vesicles. If the vesicles are disrupted, the concentration of the quencher will be greatly diluted, and the fluorescence intensity of ANTS will increase. An aliquot of the ANTS/DPX-containing vesicles was added to the peptide solution in Pipes buffer (20 mM Pipes/85 mM NaCl, pH 7.4) to obtain a final lipid concentration of 200 μM in the cuvette. The fluorescent intensities I_F were measured on a PC1 Photon Counting Fluorescence Spectrophotometer (ISS), with a $\lambda_{\text{ex}} = 353\ \text{nm}$ and a $\lambda_{\text{em}} = 525\ \text{nm}$. Slits of 1.0 and 2.0 nm were used for the λ_{ex} and λ_{em} respectively. Two neutral-density filters, 0.3 and 0.5, were placed in front of the sample to minimize photo-bleaching, and a kv 389 filter was placed in front of the detector to minimize the effect of the scattered light. The sample compartment was maintained at 24 °C with a circulating water bath. The background fluorescent intensity I_B was determined by measuring the fluorescence intensity of buffer solutions containing only the ANTS/DPX-entrapped vesicles and buffer solutions containing only the peptides. The total fluorescence intensity I_T was determined by measuring the fluorescence intensity after the vesicles were completely disrupted by 0.1 % Triton X-100. The percentage of released ANTS was calculated by the following equation: % release = $100 (I_F - I_B)/(I_T - I_B)$. Care was taken to avoid commonly encountered artifacts, such as the inner filter effect. Melittin was used as the positive control.

RESULTS

Secondary structure of NSP4 peptides

The secondary structure and interaction with model membranes of several NSP4 peptides overlapping the 114–135 enterotoxin peptide were determined. NSP4_{114–135} contains five positively charged amino acids and five negatively charged amino acids, with an overall neutral charge. Peptide NSP4_{131K} has an identical amino acid sequence as NSP4_{114–135} except that Tyr-131 was replaced with the positively charged amino acid lysine, which renders the peptide one net positive charge. The peptide D-NSP4_{114–135} has the identical NSP4_{114–135} as the amino acid sequence except that all the amino acids are D-isomers. NSP4_{120–147} is a longer peptide, the first 16 amino acids (120–135) overlap with NSP4_{114–135}, and then extend to 12 more amino acids towards the C-terminus. It has four positively charged amino acids and seven negatively charged amino acids yielding three net negative charges.

Program 'nnpredict' was used to predict the secondary structures of the whole protein and the peptides. This program uses a two-layer, feed-forward neural network to predict the secondary structure type for each residue in an amino acid sequence [29,30]. When the whole protein sequence was analysed, it predicted that amino acid residues 95–131 would adopt an α -helical structure (Table 1, only amino acids 114–147 are shown), similar to that seen in the crystal structure of residues 95–137 [31]. When the sequences of NSP4_{114–135} and NSP4_{131K} were analysed separately, both peptides were predicted to fold as an α -helical structure (Table 1). Thus predictive algorithms indicate that substitution of Tyr-131 with lysine would have no effect on the helical

Name of peptide	Predicted secondary structure*	% α -helix based on CD†		Diarrhoea induction‡	
		Buffer	50 % TFE	Dose (nmol)	% diarrhoea
NSP4 protein	HHHHHHHHHHHHHHHHHHHH-----HHHH			1.0	100 %§ (10/10)
NSP4 ₁₁₄₋₁₃₅	---HHHHHHHHHHHHHHHHHHHH--	37.0 $\pm$ 0.7	90.5 $\pm$ 1.1	50	50 % (5/10)
NSP4 _{131K}	---HHHHHHHHHHHHHHHHHHHH--	32.7 $\pm$ 0.3	72.2 $\pm$ 0.5	50	0 %§
D-NSP4 ₁₁₄₋₁₃₅		36.9 $\pm$ 0.3	91.7 $\pm$ 0.8	50	0 %
NSP4 ₁₂₀₋₁₄₇	-HHHHHHHHHHHHHHHHHHHH-----	16.8 $\pm$ 0.3	51.3 $\pm$ 0.2	50	60 % (6/10)

§ From Ball et al. [4].

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Figure 2 CD spectra of NSP4 peptides in the presence of buffer only (●), and in the presence of SUV with increasing negatively charged phospholipid DOPS contents; POPC/cholesterol/DOPS at molar ratios of 65:35:0 (○), 55:35:10 (▼) and 35:35:30 (▽)

(A) NSP4₁₁₄₋₁₃₅, (B) NSP4_{131K}, (C) D-NSP4₁₁₄₋₁₃₅ (mirror images) and (D) NSP4₁₂₀₋₁₄₇.

membranes were made with POPC (a neutral lipid), cholesterol (a sterol) and DOPS (a negatively charged lipid). The effect of lipid composition was studied by using model membranes with different concentrations of DOPS and cholesterol. To study the effect of the radius of curvature, two types of model membranes were utilized, SUV and LUV. The size of the membrane vesicles was measured by photon correlation spectroscopy, and varied between 20 and 30 nm diameter for SUVs and 100 and 120 nm

diameter for LUVs, in agreement with previous determinations [23].

Secondary structural changes in NSP4 peptide after interaction with model membranes

Even though it is difficult to use CD alone to determine the exact secondary structure of a protein or peptide, it is a very useful tool to monitor protein or peptide secondary structural changes after ligand binding or changes in structure that occur with temperature or pH alterations. In the present study, CD was utilized to monitor the secondary structural changes of NSP4 peptides after binding to membrane vesicles of different lipid composition and curvature.

Effect of negatively charged phospholipids

In the presence of neutral SUVs (POPC/cholesterol = 65:35), CD spectra of NSP4₁₁₄₋₁₃₅, NSP4_{131K}, D-NSP4₁₁₄₋₁₃₅ and NSP4₁₂₀₋₁₄₇ were almost identical with those in buffer only (Figure 2, open circles versus dark circles). Calculations based on θ values at 222 nm showed < 10 % increase in α -helical content for all the peptides compared with that in buffer only (Table 2), indicating a lack of interaction with these neutral vesicles.

In the presence of SUVs containing 10 % negatively charged DOPS, the CD spectra of NSP4 peptides changed significantly from that in buffer only or in the presence of neutral vesicles (Figure 2, filled triangle versus circles). The θ values at 222 and 208 nm became more negative, whereas they were more positive at 190 nm, indicating an increase in the α -helical content of the secondary structure in the peptide. Calculations based on θ values at 222 nm gave estimated α -helical contents of 62 % for NSP4₁₁₄₋₁₃₅, 61 % for NSP4_{131K}, 59 % for D-NSP4₁₁₄₋₁₃₅ and 32 % for NSP4₁₂₀₋₁₄₇ (Table 2). The secondary structural alterations after binding to negatively charged lipid vesicles of NSP4_{131K} and D-NSP4₁₁₄₋₁₃₅ are very similar to that of NSP4₁₁₄₋₁₃₅. For NSP4₁₂₀₋₁₄₇, even though the absolute value for α -helical content is less, the fold increases in α -helical content are comparable with those of NSP4₁₁₄₋₁₃₅. In the presence of negatively charged lipid vesicles, the α -helical contents of NSP4 peptides were 1.7, 1.9, 1.6 and 1.9 times of that in buffer only for NSP4₁₁₄₋₁₃₅, NSP4_{131K}, D-NSP4₁₁₄₋₁₃₅ and NSP4₁₂₀₋₁₄₇ respectively, indicating an interaction.

When the negatively charged DOPS content of the SUVs was increased to 30 %, the CD spectra of NSP4 peptides changed even

Table 2 Estimated α -helical content of NSP4 peptides after interaction with lipid vesicles

ND, not done.

Lipid composition of vesicles (POPC/cholesterol/DOPS)	% α -helix of NSP4 peptides*							
	NSP4 ₁₁₄₋₁₃₅		NSP4 _{131K}		D-NSP4 ₁₁₄₋₁₃₅		NSP4 ₁₂₀₋₁₄₇	
	SUV	LUV	SUV	LUV	SUV	LUV	SUV	LUV
Effect of DOPS %								
65:35:0	38.3 $\pm$ 0.1	38.4 $\pm$ 1.5	36.5 $\pm$ 0.5	32.1 $\pm$ 0.1	37.8 $\pm$ 0.6	35.9 $\pm$ 0.8	17.5 $\pm$ 0.2	16.7 $\pm$ 0.3
55:35:10	61.9 $\pm$ 0.4	40.1 $\pm$ 1.4	60.9 $\pm$ 1.5	37.3 $\pm$ 0.2	59.3 $\pm$ 0.4	38.7 $\pm$ 0.4	31.8 $\pm$ 0.5	16.6 $\pm$ 0.1
35:35:30	73.5 $\pm$ 2.2	44.1 $\pm$ 0.7	65.7 $\pm$ 1.7	48.7 $\pm$ 0.4	68.0 $\pm$ 0.8	43.4 $\pm$ 0.3	35.2 $\pm$ 0.5	17.2 $\pm$ 0.3
Effect of cholesterol %								
90:0:10	51.2 $\pm$ 0.7	39.3 $\pm$ 0.9	51.2 $\pm$ 0.1	40.0 $\pm$ 0.6	49.3 $\pm$ 0.2	38.4 $\pm$ 0.5	20.5 $\pm$ 0.3	16.8 $\pm$ 0.4
70:20:10	53.8 $\pm$ 0.4	ND	55.7 $\pm$ 0.4	ND	52.7 $\pm$ 0.2	ND	31.0 $\pm$ 0.2	ND
55:35:10	62.7 $\pm$ 0.3	40.1 $\pm$ 1.4	59.4 $\pm$ 0.6	37.3 $\pm$ 0.2	59.3 $\pm$ 0.4	39.8 $\pm$ 0.5	31.8 $\pm$ 0.5	16.1 $\pm$ 0.2
40:50:10	65.4 $\pm$ 0.9	44.6 $\pm$ 0.8	61.1 $\pm$ 0.4	37.4 $\pm$ 0.2	60.4 $\pm$ 0.6	40.6 $\pm$ 0.1	34.8 $\pm$ 0.1	16.2 $\pm$ 0.3

* Results are presented as means $\pm$ S.D. ($n = 3$ or 4).

Figure 3 CD spectra of NSP4 peptides in the presence of buffer only (●), and in the presence of SUV with 10% DOPS and increasing amount of cholesterol; POPC/cholesterol/DOPS at molar ratios of 90:0:10 (○), 70:20:10 (▼), 55:35:10 (▽) and 40:50:10 (■).

(A) NSP4₁₁₄₋₁₃₅, (B) NSP4_{131K}, (C) D-NSP4₁₁₄₋₁₃₅ (mirror images) and (D) NSP4₁₂₀₋₁₄₇.

more from that in buffer only or in the presence of neutral vesicles (Figure 2, open triangles versus circles). The θ values at 222 and 208 nm became more negative, and were more positive at 190 nm, showing a greater increase in α -helical content of the secondary structure of the peptide. Calculations based on θ values at 222 nm gave estimated α -helical contents of 74% for NSP4₁₁₄₋₁₃₅, 66% for NSP4_{131K}, 68% for D-NSP4₁₁₄₋₁₃₅ and 35% for NSP4₁₂₀₋₁₄₇ (Table 2). The α -helical contents of NSP4 peptides were 2.0, 2.0, 1.8 and 2.1 times of that in buffer only for NSP4₁₁₄₋₁₃₅, NSP4_{131K}, D-NSP4₁₁₄₋₁₃₅ and NSP4₁₂₀₋₁₄₇ respectively.

In summary, NSP4 peptides preferentially interact with anionic SUVs based on secondary structural changes after interaction, and become more α -helical with an increase in negatively charged lipids.

Effect of cholesterol

First, as seen above, in the presence of SUVs that contained 35% cholesterol and no negatively charged lipid, CD spectra and α -helical content of the NSP4 peptides were almost identical with that in buffer only (Figure 2, open circles versus black circles). These results indicate that cholesterol alone is insufficient for optimal binding between the C-terminal NSP4 peptides and lipid vesicles.

Secondly, in the presence of SUVs that contained 10% DOPS and no cholesterol, the CD spectra of NSP4 peptides changed when compared with buffer only (Figure 3, open circles versus black circles). The α -helical content of the peptide NSP4₁₁₄₋₁₃₅ was 51% (Table 2) ($P < 0.01$, significantly different from that in buffer) or 1.4 times of that in buffer only, indicating that cholesterol was not absolutely necessary for NSP4₁₁₄₋₁₃₅ to bind the lipid vesicles. Similarly, in the presence of SUVs containing 10% DOPS and no cholesterol, the α -helical contents of NSP4_{131K},

Figure 4 CD spectra of NSP4 peptides in the presence of buffer only (●), and in the presence of LUV with increasing negatively charged phospholipid DOPS contents; POPC/cholesterol/DOPS at molar ratios of 65:35:0 (○), 55:35:10 (▼) and 35:35:30 (▽).

(A) NSP4₁₁₄₋₁₃₅, (B) NSP4_{131K}, (C) D-NSP4₁₁₄₋₁₃₅ (mirror images) and (D) NSP4₁₂₀₋₁₄₇.

D-NSP4₁₁₄₋₁₃₅ and NSP4₁₂₀₋₁₄₇ were 51, 49 and 21% (Table 2) ($P < 0.01$, significantly different from that in buffer) or 1.6, 1.3 and 1.2 times of that in buffer only respectively.

Thirdly, when cholesterol was increased to 20, 35 or 50% in the SUVs while the percentage of DOPS was maintained at 10%, significant changes in the CD spectra (increased α -helical content) were observed (Figure 3, filled and open triangles and filled squares). Calculations based on θ values at 222 nm gave estimated α -helical contents of NSP4₁₁₄₋₁₃₅ of 54, 63 and 65% (Table 2) ($P < 0.01$, all significantly different from that in buffer), when the cholesterol contents of the SUVs were increased to 20, 35 and 50% respectively. Similarly, the α -helical contents of NSP4_{131K} were increased to 56, 59 and 61%, the α -helical contents of D-NSP4₁₁₄₋₁₃₅ were increased to 53, 59 and 60% ($P < 0.01$, all significantly different from that in buffer) and the α -helical contents of NSP4₁₂₀₋₁₄₇ increased to 31, 32 and 35% (Table 2) ($P < 0.01$, all significantly different from that in buffer). At the highest SUV cholesterol content studied (50%), the α -helical contents of NSP4 peptides were 1.8, 1.9, 1.6 and 2.1 times of that in buffer only for NSP4₁₁₄₋₁₃₅, NSP4_{131K}, D-NSP4₁₁₄₋₁₃₅ and NSP4₁₂₀₋₁₄₇ respectively.

Effect of membrane curvature

As indicated above, NSP4 peptides interacted much more strongly with SUVs when the SUVs were rich in negatively charged phospholipids and cholesterol. Next, the same experiments were performed with LUVs to evaluate the effect(s) of membrane curvature. When the percentages of negatively charged lipid DOPS were increased from 0 to 30%, only NSP4_{131K} showed a significant change in structure as observed in the CD spectrum (Figure 4B). Calculations showed the secondary structure changed

Figure 5 CD spectra of NSP4 peptides in the presence of buffer only (●), and in the presence of LUV with 10% DOPS and increasing amount of cholesterol; POPC/cholesterol/DOPS at molar ratios of 90:0:10 (○), 55:35:10 (▼) and 40:50:10 (▽)

(A) NSP4₁₁₄₋₁₃₅, (B) NSP4_{131K}, (C) D-NSP4₁₁₄₋₁₃₅ (mirror images) and (D) NSP4₁₂₀₋₁₄₇.

from 32% α -helix with neutral LUV to 37% α -helix ($P < 0.01$, significantly different from that in buffer) with 10% DOPS LUV to 49% α -helix ($P < 0.01$, significantly different from that in buffer) with 30% DOPS LUV. This alteration is probably due to the net positive charge of NSP4_{131K}, which promotes its interaction with negatively charged surfaces. However, these structural changes were small when compared with the interactions with SUVs. For NSP4₁₁₄₋₁₃₅, D-NSP4₁₁₄₋₁₃₅ and NSP4₁₂₀₋₁₄₇, there was very little secondary structural changes after interaction with LUVs containing 35% cholesterol and up to 30% DOPS (Figure 4 and Table 2).

CD spectra of each NSP4 peptide were analysed in the presence of LUVs containing 10% DOPS and increasing amount of cholesterol (0, 20, 35 and 50%). As shown in Figure 5 and Table 2, all of the peptides underwent very little or no secondary structural alterations after interaction. This lack of secondary structural changes could be due to a lack of partitioning or due to partitioning without secondary structural changes. An independent filtration assay (partitioning assay) was performed to differentiate between these two possibilities.

NSP4 peptide interactions with model membranes: a filtration-partitioning assay

To confirm the interaction of the C-terminal region of NSP4 peptides with SUVs and to ensure that partitioning was absent when the secondary structure was unaltered, each peptide was incubated with lipid vesicles, and the partitioned versus free peptide was separated by filtration through a membrane with a molecular mass cut-off of 100 kDa. The unbound, free peptides in the flow-through were quantified by an *o*-phthalaldehyde assay.

Figure 6 NSP4 peptides binding to SUV (A) and LUV (B)

Filled columns, NSP4₁₁₄₋₁₃₅; hatched columns, NSP4_{131K}; open columns, D-NSP4₁₁₄₋₁₃₅; gridded columns, NSP4₁₂₀₋₁₄₇. Results presented are means $\pm$ S.E.M. ($n = 4$).

After incubation with neutral SUV (POPC/cholesterol = 65:35), approx. 100% of the NSP4 peptides were in the flow-through fraction. Thus there was no detectable partitioning to the lipid vesicles (Figure 6). After interaction with negatively charged SUVs with a high cholesterol content, (POPC/cholesterol/DOPS = 40:50:10), the percentages of free peptide decreased to 62, 56, 82 and 57% ($P < 0.01$, all significantly different from that with neutral vesicles) for NSP4₁₁₄₋₁₃₅, NSP4_{131K}, D-NSP4₁₁₄₋₁₃₅ and NSP4₁₂₀₋₁₄₇ respectively, which indicated that 18–44% of the peptides partitioned to the lipid vesicles. When the peptides were incubated with SUVs with an increased negative charge (POPC/cholesterol/DOPS = 35:35:30), the percentage of free peptide further decreased to 19, 11, 18 and 28% ($P < 0.01$, all significantly different from that with neutral vesicles) for NSP4₁₁₄₋₁₃₅, NSP4_{131K}, D-NSP4₁₁₄₋₁₃₅ and NSP4₁₂₀₋₁₄₇ respectively, which indicated that 72–89% of the peptides were partitioned to the lipid vesicles (Figure 6). Thus the secondary structural alterations of the peptide after membrane interaction, as measured by CD, correlate with partitioning to the SUVs.

The partitioning of the NSP4 peptides with LUVs is shown in Figure 6(B). In the presence of neutral or negatively charged LUVs (POPC/cholesterol = 65:35 and POPC/cholesterol/DOPS = 40:50:10), approx. 100% of the NSP4 peptides were in the flow-through fraction, indicating no detectable partitioning to the lipid vesicles. When the percentage of DOPS was increased to 30% (i.e. POPC/cholesterol/DOPS = 35:35:30), the percentages of free peptide decreased to 67, 39, 76 and 76% ($P < 0.01$, all significantly different from that with neutral vesicles) for NSP4₁₁₄₋₁₃₅, NSP4_{131K}, D-NSP4₁₁₄₋₁₃₅ and NSP4₁₂₀₋₁₄₇ respectively. The percentage of peptide partitioned to negative LUVs was much less ($P < 0.01$) than that partitioned to the negative SUVs of the

Table 3 PS distribution of lipid vesicles of different sizes and lipid compositions

Lipid composition of the vesicles†	PS distribution (outer leaflet/total) Means $\pm$ S.E.M. ($n = 4$)		[PS] _{out} ‡ (mM)	
	SUV	LUV	SUV	LUV
90:0:10	0.689 $\pm$ 0.006*	0.481 $\pm$ 0.008	0.069	0.048
55:35:10	0.710 $\pm$ 0.004	0.473 $\pm$ 0.008	0.071	0.047
40:50:10	0.661 $\pm$ 0.005**	0.409 $\pm$ 0.004**	0.066	0.041
35:35:30	0.628 $\pm$ 0.005**	0.369 $\pm$ 0.005**	0.188	0.111

Significantly different from that of vesicles containing POPC/cholesterol/DOPS at a molar ratio of 55:35:10. * $P < 0.05$; ** $P < 0.01$.

† Lipid compositions of vesicles are presented as mole ratio of POPC/cholesterol/DOPS.

‡ Calculated outer leaflet PS concentrations at total lipid concentration 1 mM used in CD measurements.

same lipid composition. In agreement with the CD analyses, more NSP4_{131K} was partitioned to the negative LUVs than the other peptides, probably because of its net positive charge. However, there was significant partitioning of all the peptides to LUV containing 30% DOPS (POPC/cholesterol/DOPS = 35:35:30) (Figure 6B), and very little secondary structural changes in the peptide (except for NSP4_{131K}) (Figure 4), indicating that these peptides underwent less secondary structural changes after interaction with LUV when compared with SUV.

PS transbilayer distribution of the model membranes

To extend the observation that the C-terminal NSP4 peptides preferentially interacted with lipid bilayers that are rich in negatively charged phospholipid, high in cholesterol and have a high radius of curvature, the transbilayer distribution of the DOPS and how it is affected by lipid composition and size of the vesicles were determined by a TNBS assay. The different compositions of the vesicles were compared with the vesicles that were composed of POPC/cholesterol/DOPS = 55:35:10 (Table 3).

SUVs with lipid composition POPC/cholesterol/DOPS = 55:35:10 were found to have 71% of the DOPS in the outer leaflet ([PS]_{out} = 0.071 mM; Table 3). It is known that for SUVs, approx. 70% of the lipids are in the outer leaflet due to the high membrane curvature [33], therefore, at this lipid composition, DOPS has no preference for inner or outer leaflet. When the SUV cholesterol content was decreased to 0%, the distribution of DOPS remained essentially the same (Table 3). The percentage of DOPS in the outer leaflet decreased to 66.1% when the SUV cholesterol content was increased to 50% (Table 3). When the SUV DOPS content increased to 30%, DOPS in the outer leaflet decreased to 62.8%, however [PS]_{out} increased to 0.188 mM.

LUVs (POPC/cholesterol/DOPS = 55:35:10) had 47.3% of the DOPS in the outer leaflet ([PS]_{out} = 0.047 mM), which is very close to the known total lipid distribution of LUVs (50% in the outer leaflet) [34]. As with the SUVs, when the LUV cholesterol content decreased to 0%, the PS distribution was about the same (Table 3), whereas, when the LUV cholesterol content was increased to 50%, the percentage of DOPS in the outer leaflet decreased to 40.9% (Table 3). When the LUV DOPS content increased to 30%, the percentage of DOPS in the outer leaflet decreased to 36.9% and [PS]_{out} increased to 0.111 mM.

Results from the TNBS assay showed the following: (i) Increasing the DOPS content from 10 to 30% resulted in a higher percentage of PS in the inner leaflet of both the SUVs and LUVs. However, the overall DOPS concentration in the outer

Table 4 Results of particle size analysis of SUV and LUV in the presence and absence of NSP4 114–135

Lipid compositions of SUV and LUV were POPC/cholesterol/DOPS at a molar ratio of 35:35:30.

Sample	Mean particle size $\pm$ S.D. (nm)
SUV	19.4 $\pm$ 4.4
SUV + NSP4 ₁₁₄₋₁₃₅	18.7 $\pm$ 4.3
LUV	101.0 $\pm$ 20.3
LUV + NSP4 ₁₁₄₋₁₃₅	101.0 $\pm$ 21.9

leaflet increased, which probably accounts for the stronger peptide interaction with vesicles containing 30% DOPS. (ii) When the cholesterol content was increased from 0 to 35% in the SUVs, the PS distribution remained the same. However, when the cholesterol content increased from 35 to 50%, the PS concentration in the outer leaflet decreased. Given that there were stronger peptide interactions at the higher cholesterol concentration, and that the PS in the outer leaflet either was unaltered or decreased with increasing cholesterol, it appears that other factors outside of the PS content in the outer leaflet contributed to the peptide interactions. One possible explanation is that cholesterol induces the formation of DOPS patches or microdomains rich in negative charge. (iii) When SUVs and LUVs with the same lipid composition were compared, the SUVs always had a higher DOPS content in the outer leaflet. This is probably one of the reasons that the peptides interacted more strongly with negative SUV when compared with LUV. However, LUVs with 30% PS have more [PS] on the outer leaflet when compared with SUVs with 10% PS, but the peptides still interacted more strongly with the SUVs with 10% PS than the LUVs with 30% PS (CD data, Table 2). These results support the idea that other factors, such as lipid packing density or membrane curvature, contribute to the peptide–lipid vesicle interactions.

Size of SUV and LUV in the presence of NSP4₁₁₄₋₁₃₅

The particle size distribution of the SUVs and LUVs in the presence and absence of NSP4₁₁₄₋₁₃₅ was measured using a Coulter N4 Plus Photon Correlation Spectrometer. In the absence of NSP4₁₁₄₋₁₃₅, the mean size of the SUVs and LUVs was 19 and 101 nm respectively (Table 4). In the presence of NSP4₁₁₄₋₁₃₅, the mean particle size and size distribution of both the SUVs and LUVs were unaltered (Table 4), indicating that the peptide did not induce membrane fusion or other processes that would lead to changes in the vesicle mean size or size distribution.

Liposome leakage assay

Leakage assays were performed to determine if any of the NSP4 peptides caused vesicle disruption. The vesicles (both SUV and LUV) were prepared with POPC/cholesterol/DOPS at a molar ratio of 55:35:10 and entrapped with the fluorophore/quencher pair ANTS/DPX. The first leakage assays were performed with the LUVs (final lipid concentration was kept at 200 μ M). Melittin, a peptide known to disrupt membranes, was used as a positive control. At 1 μ M concentration, melittin disrupted the vesicles resulting in leakage of the fluorescent dye (Figure 7, black squares). The leakage reached a maximum in approx. 25 min with 88% of the entrapped materials released. When the melittin concentration was increased to 2.5 μ M, 99% of the entrapped dye was released in 10 min (Table 5). Under similar conditions,

Figure 7 Fluorescent dye release from LUV (POPC/cholesterol/DOPS = 55:35:10)

The release of entrapped ANTS/DPX from LUV (200 μ M) was measured in the presence of: ■, melittin (1.0 μ M); ▲, NSP4_{131K} (100 μ M); ○, NSP4₁₁₄₋₁₃₅ (100 μ M); and ●, NSP4₁₂₀₋₁₄₇ (100 μ M).

Table 5 Leakage assay with ANTS/DPX-containing lipid vesicles

	% release (means $\pm$ S.E.M.)	
	LUV*	SUV*
Melittin		
2.5 μ M	99.2 $\pm$ 0.2 (<i>n</i> = 3)	64 $\pm$ 3 (<i>n</i> = 3)
5 μ M		100 $\pm$ 2 (<i>n</i> = 3)
NSP4 ₁₁₄₋₁₃₅		
15 μ M	0.6 $\pm$ 0.5 (<i>n</i> = 3)	
100 μ M	1.1 $\pm$ 0.6 (<i>n</i> = 3)	−0.6 $\pm$ 3 (<i>n</i> = 3)
NSP4 ₁₂₀₋₁₄₇		
15 μ M	−0.6 $\pm$ 0.5 (<i>n</i> = 3)	
100 μ M	1.6 $\pm$ 1 (<i>n</i> = 2)	0.7 $\pm$ 1.5 (<i>n</i> = 2)
NSP4 _{131K}		
15 μ M	0.5 $\pm$ 0.5 (<i>n</i> = 3)	
100 μ M	18.1 $\pm$ 0.5 (<i>n</i> = 2)	2.3 $\pm$ 1.1 (<i>n</i> = 2)

* The leakage assay was performed at vesicle lipid concentration 200 μ M (as determined by phosphorus assay). The lipid compositions of SUV and LUV were POPC/cholesterol/DOPS at a molar ratio of 55:35:10.

but at a peptide concentration of 15 μ M, none of the NSP4 peptides (NSP4₁₁₄₋₁₃₅, NSP4₁₂₀₋₁₄₇ and NSP4_{131K}) caused dye release (Table 5). At a peptide concentration of 100 μ M (100-fold greater concentration than melittin), NSP4_{131K} caused 18% of the entrapped dye to be released into the media (Figure 7, black triangles and Table 5). Even at the 100 μ M concentration, no leakage was observed with NSP4₁₁₄₋₁₃₅ or NSP4₁₂₀₋₁₄₇ (Table 5).

Leakage of ANTS/DPX-containing SUV (final lipid concentration was maintained at 200 μ M) was then tested. Melittin, a peptide with known membrane destabilization activity, was used as the positive control. At 2.5 μ M, melittin disrupted the vesicles and 64% of the dye was released, and at a melittin concentration of 5 μ M, 100% of the entrapped dye was released (Table 5). The same leakage experiments were performed with NSP4₁₁₄₋₁₃₅, NSP4₁₂₀₋₁₄₇ and NSP4_{131K}. No significant leakage was observed with any of the peptides when tested at a peptide concentration up to 100 μ M (Table 5). Therefore the peptides tested did not induce membrane disruption or disorganization after interaction.

DISCUSSION

The discovery that NSP4₁₁₄₋₁₃₅ functions as an enterotoxin similar to the intact protein, albeit less actively [4], together with the secretion of NSP4₁₁₂₋₁₇₅ [14], which contains residues 114–135, suggests the importance of this region. Available evidence indicates that extracellular NSP4₁₁₂₋₁₇₅ interacts with a receptor on neighbouring cells to trigger a calcium-signalling pathway resulting in diarrhoea [14]. Previously, NSP4 was shown to mobilize $[Ca^{2+}]_i$ concentration via phospholipase C activation and inositol triphosphate production in cultured intestinal cells [7], and to mimic the cholinergic agonist, carbachol, by potentiating cAMP-mediated secretory ion (Cl^-) transport across mouse unstripped intestinal mucosa [4]. NSP4₁₁₄₋₁₃₅ also appears to function as a non-competitive inhibitor of the Na^+ -D-glucose symporter 1 (SGLT1) [35], such that sodium-coupled water reabsorption across intestinal membranes could be inhibited by the enterotoxic peptide. In this model, the released NSP4₁₁₂₋₁₇₅ would promote this anti-absorptive effect by binding neighbouring cell receptors on apical intestinal membranes. Our results showed that the 114–135 peptide fails to promote membrane destabilization, supporting these studies and predicting that NSP4 interacts with membrane receptors.

The activity of NSP4 and the 114–135 peptide are CFTR (cystic fibrosis transmembrane conductance regulator)-independent; diarrhoea is induced in CFTR-depleted (null mutation) neonatal mice when administered with either the protein or the peptide [36]. Furthermore, examination of isolated large and small intestinal crypt cells from normal and CFTR null mice reveal that NSP4-induced $[Ca^{2+}]_i$ mobilization is independent of cell differentiation and/or crypt age and does not regulate the age-dependent chloride secretion. Rather, NSP4 elicits an age- and Ca^{2+} -dependent anion plasma membrane permeability as measured by I^- influx [36]. The authors conclude that the primary functions of NSP4 may be to activate an apical membrane Ca^{2+} -dependent Cl^- secretion in mouse pup enterocytes and to facilitate CFTR-mediated fluid transport by up-regulating Ca^{2+} -sensitive basolateral Cl^- uptake [36].

Protein–membrane interactions are known to function in many cellular events such as signal transduction, membrane transport and protein trafficking. Previously, we reported that NSP4 and NSP4₁₁₄₋₁₃₅ preferentially bind caveolae-like membranes, i.e. membranes rich in negatively charged phospholipids and cholesterol, and have a high radius of curvature [23]. The preferential interaction of NSP4 with caveolae-like membranes is reasonable given that caveolae function as a focal point for the signalling machinery of the cell and form a unique endocytic and exocytic compartment at the cell surface [37]. In addition, several findings suggest a role for caveolae and cholesterol interactions during Ca^{2+} signalling events. Ca^{2+} ATPase, inositol triphosphate receptors, tyrosine kinases, G-proteins and calmodulin, key molecular components of Ca^{2+} regulation, have been localized to caveolae [37–39]. As much as 50% of cellular phosphatidylinositol 4,5-bisphosphate compartmentalizes to caveolae and caveolae are the primary source of phosphatidylinositol 4,5-bisphosphate hydrolysis in response to a ligand, such as NSP4 [40]. Thus a specific interaction of NSP4 with receptors localized to caveolae would induce the biological functions of the toxic protein.

The results of this study showed that NSP4₁₁₄₋₁₃₅-related peptides interact with membranes comprised only of lipids (POPC, cholesterol and DOPS), in the absence of other types of receptor molecules (e.g. glycoprotein or glycolipid). The membrane interactions were confirmed by two independent assays. CD experiments revealed that the peptides underwent significant secondary

structural changes after membrane interaction, and the filtration-binding assays showed the partitioning of the peptides to the membranes. Hence, the NSP4 peptides are membrane-active. The secondary structural alterations of NSP4 and its peptides after interacting with membrane vesicles suggest that a similar change would occur *in vivo*. We propose that the increased helical content of the NSP4 enterotoxigenic region, after interacting with caveolae microdomains, places the receptor-binding residues in a more advantageous conformation for binding of the cell receptor or, alternatively, exposes the receptor-binding domain for interaction with the receptor. Additional studies are needed to test this hypothesis.

NSP4_{131K} was the only peptide known to bind significantly with negatively charged LUVs, and to have significant secondary structural changes after interaction with anionic LUVs. The additional positive charge of NSP4_{131K} increased interactions with negatively charged membranes and reduced selectivity towards highly curved membrane vesicles. In contrast, changing the orientation of all the amino acids in NSP4₁₁₄₋₁₃₅ from L- to D-configuration (D-NSP4₁₁₄₋₁₃₅) did not alter the α -helical content, nor did it change binding to the negatively charged membranes. NSP4₁₂₀₋₁₄₇ has a shorter α -helical region when compared with NSP4₁₁₄₋₁₃₅, yet bound equally well to the membrane vesicles. Neither NSP4_{131K} nor D-NSP4₁₁₄₋₁₃₅ induced diarrhoea in neonatal mice [4] and the NSP4_{131K} peptide failed to inhibit SGLT1 [35].

The lipid vesicle leakage assays showed that none of the peptides induced significant leakage of fluorescent dyes at physiological concentrations. At a concentration of 100 μ M (100-fold greater than the concentration of melittin), NSP4_{131K} induced 18% dye release. Since NSP4_{131K} failed to promote calcium release or cause diarrhoea, its ability to induce leakage at a high concentration may be due to a detergent effect and is probably not relevant. Vesicle size distribution analyses performed with and without NSP4₁₁₄₋₁₃₅ showed that the peptide did not change the size or size distribution of the membrane vesicles, indicating residues 114–135 do not cause membrane fusion or disruption. NSP4₁₁₄₋₁₃₅ and NSP4₁₂₀₋₁₄₇ failed to evoke non-specific membrane disruption or membrane fusion, in contrast with previously reported results, which showed destabilization of ER-like vesicles [21]. However, the procedures and concentrations of peptide were different from those used in the present study. In addition, the LUVs previously utilized were not characterized and appeared as both LUVs and SUVs by electron microscopy, nor were neutral density filters employed, any one of which could explain the different results. A recent study [15] demonstrated that the amino acids 48–91 of NSP4 possesses membrane-destabilizing activity, whereas NSP4₁₁₄₋₁₃₅ by itself has no effect on membrane stability, but enhances the membrane-destabilizing activity of residues 48–91 when both peptides are present.

The peptides' preference for negatively charged membranes suggests an electrostatic component to the binding. However, this interaction was not solely due to the degree of anionic phospholipid exposure in the outer leaflet. Increasing the content of anionic phospholipid in the outer leaflet of the low-curvature membranes LUVs to the same extent or more than in SUV, resulted in much weaker interactions as indicated by less secondary structural changes and less partitioning to the membrane vesicles. NSP4 peptide interactions with anionic SUV membranes were potentiated by the presence of cholesterol. A previous study proposed a putative cholesterol recognition/interaction motif, -L/V-(X)₁₋₅-Y-(X)₁₋₅-R/K- [41]. This amino acid pattern is contained in all the peptides examined in the present study, except for NSP4_{131K}. Increased cholesterol content in neutral lipid membranes had no effect on the NSP4 peptide-membrane interactions, whereas increased cholesterol content in membranes

containing 10% DOPS enhanced the peptides' partitioning with membranes. The increased cholesterol content in the negatively charged vesicles failed to increase the percentage distribution of DOPS in the outer leaflet. These results suggest that the increased cholesterol content may induce a change in PS distribution (not concentration) within the leaflets, such as the formation of microdomains rich in negatively charged lipids and/or cholesterol. Previous studies demonstrate phase separations of membranes containing PS and cholesterol [42,43]. Furthermore, the formation of these microdomains might be responsible for the increased NSP4 peptide membrane partitioning.

The preferential interaction of the NSP4 peptides with highly curved membranes has precedence in other membrane-binding proteins and peptides [44,45]. The structural basis of NSP4 peptide interaction with highly curved membranes includes the more mobile packing and lower surface pressure of the phospholipids in the outer leaflet of SUV [44,45], lower packing constraints in the outer leaflet [46] and more phospholipid packing defects in the SUV outer leaflet to allow insertion of proteins/peptides [47].

Although all of the peptides tested bound lipid membranes, NSP4_{131K} and D-NSP4₁₁₄₋₁₃₅ failed to induce diarrhoea in neonatal mouse pups. NSP4₁₁₄₋₁₃₅ and NSP4₁₂₀₋₁₄₇ induced diarrhoea in 50 and 60% of the mice tested respectively; these active peptides bound equally well to highly curved membrane vesicles rich in negatively charged lipids and cholesterol. In contrast, the additional positive charge in NSP4_{131K} resulted in stronger interactions with both negative SUVs and LUVs. NSP4₁₁₄₋₁₃₅ and D-NSP4₁₁₄₋₁₃₅ interacted with the caveolae-like membranes equally well, indicating that the orientation of the α -helix had no effect on its interaction with membranes. Although a direct correlation between diarrhoea induction and membrane binding is not obvious, the NSP4_{131K} may fail to target highly curved membrane domains or may bind alternate membrane receptor molecules. The α -helix orientation of D-NSP4₁₁₄₋₁₃₅ would greatly affect its interaction with receptors. The lack of diarrhoea induction by D-NSP4₁₁₄₋₁₃₅ supports the premise that NSP4 induces diarrhoea by a receptor-mediated event that promotes intracellular signalling, calcium mobilization and chloride secretory currents.

Five genotypes of NSP4 have been identified by computer analyses of currently known NSP4 sequences [48]. Conserved residues have been localized interspersed in the enterotoxigenic region as well as in other locations of the protein. There are common variable regions at residues 70–78, 91–98, 135–142 and at the C-terminus. The variable region encompassed by amino acids 135–142 varies in all genotypes [48]. Variation within this enterotoxigenic region of NSP4 may explain the different clinical outcomes of natural rotavirus infections. The different NSP4 primary sequences may have a similar secondary structure, but could vary in structural stability, interactions with caveolae-like membranes and binding of receptor molecules.

In summary, the present study showed an α -helical region that is common to peptides overlapping NSP4₁₁₄₋₁₃₅, which may function as a membrane interacting domain. We propose that this membrane-binding site selectively partitions to a lipid domain resembling caveolae or rafts resulting in secondary structural changes important for interaction with receptors located within or near the caveolae microdomain. The orientation of the α -helix and the non-helical region of NSP4₁₂₀₋₁₄₇ may similarly play important roles in binding to a specific receptor or optimally positioning the NSP4 receptor-binding domain to interact with the receptor. We were unable to correlate diarrhoea induction to structural alterations after binding defined membrane vesicles. Perhaps this lack of correlation affirms our hypothesis. The

variable residues could be a natural plasticity enabling NSP4 from different species to interact with different, species-specific receptor molecules on the cell surface, whereas the conserved amino acids maintain the helical structure to ensure that the enterotoxin domain is positioned where it interacts with the receptor localized to caveolae/rafts.

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