Membrane-destabilizing activity of rotavirus NSP4 is mediated by a membrane-proximal amphipathic domain

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Expression of the rotavirus non-structural glycoprotein NSP4 in E. coli leads to a decrease in optical density of the culture and release of [3H]uridine into the medium, effects attributable to the ability of NSP4 to perturb the bacterial membrane. To identify a domain of NSP4 responsible, different regions of the polypeptide were expressed in E. coli. Membrane destabilization is associated with a region of the protein located within residues 48–91, which includes a potential cationic amphipathic helix. A second region of NSP4 that contains a coiled-coil oligomerization domain and a sequence reported to function as a viral enterotoxin enhances the membrane-destabilizing activity of residues 48–91, but has no direct effect on the membrane stability. These studies suggest that the membrane-destabilizing and enterotoxic properties of NSP4 may be mediated by different regions of the polypeptide and suggest a possible basis for the cytotoxicity of NSP4 in mammalian cells.

Rotavirus is a leading cause of infantile gastroenteritis worldwide (reviewed in Kapikian & Channock, 1996). Virus infection of cultured cells can lead to profound morphological and biochemical alterations resulting in cell death (Estes, 1996; Michelangeli et al., 1991; Newton et al., 1997). Several of the cytopathic effects associated with rotavirus infection are also evident when a single viral polypeptide, the non-structural glycoprotein NSP4, is expressed in animal cells. Expression of NSP4 in Sf9 cells causes an increase in the concentration of cytoplasmic Ca^{2+} to levels that are potentially toxic (Tian et al., 1994, 1995). In cultured mammalian cells, expression of NSP4 using a recombinant vaccinia virus results in a loss of plasma membrane integrity, morphological changes and ultimately cell death (Newton et al., 1997).

NSP4 has been identified as a viral enterotoxin based on its ability to promote diarrhoea in infant mice when added intra- peritoneally and intra-ileally (Ball et al., 1996; Horie et al., 1999). The enterotoxic behaviour of NSP4 is mediated by its interaction with a putative plasma membrane receptor triggering a phospholipase C-mediated increase in intracellular Ca^{2+} leading to enhanced Cl^{−} secretion in epithelial cells (Dong et al., 1997; Morris et al., 1999).

The cytopathic and enterotoxic activities of NSP4 would appear to arise through different mechanisms but neither are fully understood. Given the intracellular location of this glycoprotein [NSP4 is an integral membrane protein resident in the endoplasmic reticulum (ER)], it is also unclear how NSP4 might be released into the cell medium, a likely prerequisite for enterotoxic activity. A peptide derived from the cytoplasmic domain of NSP4 (amino acids 114–135) has been shown to possess both enterotoxic activity and cause the permeabilization of liposomal membrane vesicles in vitro (Ball et al., 1996; Tian et al., 1996). Membrane destabilization activity may account for the NSP4 cytopathic effect through disruption of the ER leading to an increase in cytoplasmic Ca^{2+} concentration (Tian et al., 1996).

We have previously shown that a membrane-proximal domain of NSP4 that is rich in basic amino acid residues and distinct from the enterotoxin peptide sequence (Fig. 1) is important in mediating the cytopathic effects of NSP4 in MA104 cells (Newton et al., 1997). Modelling this amino acid sequence (residues 54–74) as an α-helix reveals the potential for a highly amphipathic structure to form in which five lysine residues are clustered on one face of the helix. Since cationic amphipathic helices are motifs common to many polypeptides with membrane-distabilizing properties (Epand et al., 1995), we predicted that this region of NSP4 might mediate disruption of the ER membrane in vivo. A direct demonstration of membrane-distabilizing activity of NSP4 in mammalian cells is hampered by the intracellular location of the protein and thus relies on indirect methods such as measurement of Ca^{2+} release from the intracellular store. Addition of purified NSP4 to ER-derived membrane vesicles represents an alternative strategy but given the integral membrane status of the protein, its extraction and solubilization require the use of detergents that would effect the permeability of membrane vesicles subsequently incubated with the purified protein. In this study we have used inducible expression of NSP4 in E. coli to examine
Fig. 1. Schematic representation of the NSP4 polypeptide sequence and the truncated domains expressed in E. coli. Shaded boxes labelled H1, H2 and H3 represent hydrophobic regions. Note that H2 is the transmembrane domain and that the cytoplasmic domain of NSP4 begins at residue 45 (Bergmann et al., 1989). The hatched box represents the coiled-coil oligomerization domain and the location of the enterotoxic peptide sequence is underlined with a broken line. Numbers refer to the respective positions within the amino acid sequence of NSP4. The position of the region predicted to form an amphipathic structure is indicated (+ + +).

Fig. 2. Expression of NSP4 and its truncated domains in E. coli BL21 (DE3) pLysS cells. Cultures of bacteria transformed with pET17xb or a recombinant plasmid containing the appropriate NSP4 cDNA fragment were grown in minimal medium supplemented with 2% glucose and induced when the optical density of the culture reached 0.8–1. At the indicated time post-induction, 1 ml aliquots of the culture were removed and incubated with 5 µCi [35S]methionine for a further 10 min. Where indicated, rifampicin (175 µg/ml) was added 10 min prior to the commencement of labelling. Total cellular protein was resolved by SDS–PAGE and labelled proteins were visualized by autoradiography. Autoradiographs were scanned individually using a Microtek Slimscan C3 and compiled using Adobe Photoshop on a Macintosh G3 computer. The arrow indicates the expected position of either full-length NSP4, the appropriate truncated species or the T7 gene protein expressed from the parental plasmid.

potential membrane destabilization and to map the region(s) within the protein that mediate this activity. Inducible expression in bacteria has proved a valid and useful system for the identification of polypeptides from animal viruses with membrane-destabilizing properties (Arroyo et al., 1995; Carrasco, 1994; Guinea & Carrasco, 1992; Sanz et al., 1994). Furthermore, the lipid composition of the bacterial inner membrane is similar to the membrane of the ER, both of
Membrane destabilization by rotavirus NSP4

which lack appreciable quantities of cholesterol, a significant component of the plasma membrane of mammalian cells.

In order to express NSP4 in E. coli, the gene encoding NSP4 from rotavirus strain SA11 (Both et al., 1983) was first cloned into the prokaryotic expression vector pET17-xb (Novagen) as a NdeI–BamHI fragment to replace the sequence encoding the bacteriophage T7 gene 10, incorporated in the parent vector. Plasmids were also constructed that expressed a single truncated NSP4 domain (Fig. 1). These were generated using standard PCR techniques employing oligonucleotide primers to insert a NdeI restriction site/AUG start codon and BamHI restriction site/stop codon at various positions within the NSP4 coding sequence. Amplified DNA fragments were cloned into pBluescript-II (Invitrogen) and sequenced to verify the cloned DNA fragment (Applied Biosystems 377A sequencer). The amplified fragments were then transferred to NdeI/BamHI-digested pET17xb to generate a series of recombinant plasmids that expressed either the full-length or a truncated NSP4 polypeptide.

Exponentially growing cultures of E. coli strain BL21(DE3) pLysS transformed with appropriate NSP4-expressing plasmids were induced by the addition of 1 mM IPTG. The host bacterial strain is a lysogen that inducibly expresses T7 RNA polymerase under the control of a lac promoter (Studier & Moffat, 1986). In addition, the host constitutively synthesizes T7 lysozyme, an inhibitor of T7 RNA polymerase activity, and thus the expression of potentially toxic gene products is tightly repressed in the uninduced state. Expression of NSP4 following induction was monitored by the labelling of polypeptides during induction with $^{[35}S$S$]$methionine, followed by SDS–PAGE and autoradiographic analysis (Fig. 2). Selective labelling of the plasmid-encoded gene products was achieved by addition of rifampicin to induced cultures 10 min prior to addition of the radiolabel. In each case, a new polypeptide species was synthesized whose apparent molecular mass was consistent with that anticipated for either the full-length NSP4 polypeptide (unglycosylated), or the relevant truncated variants (Fig. 2). Expression levels varied considerably, with peptides representing soluble regions of the cytoplasmic domain (NSP4$^{48-91}$ or NSP4$^{48-139}$) accumulating to the highest levels, while peptides containing hydrophobic domains were poorly expressed.

Two assays were utilized to examine the effect of NSP4 expression on membrane permeability in E. coli. First, the optical density of each bacterial culture was monitored after the induction of NSP4 expression. Perturbation of the bacterial inner membrane causes release of T7 lysozyme from the cell resulting in cell lysis. Expression of NSP4 resulted in a progressive decline in the optical density of cultures expressing NSP4 is due to its membrane-destabilizing activity leading to release of T7 lysozyme and cell lysis.

Expression of some of the truncated NSP4 variants also resulted in growth arrest and cell lysis. A 44 amino acid domain comprising residues 48–91 from NSP4 (NSP4$^{48-91}$) had a similar effect on growth to the full-length protein. A striking result was the much more rapid reduction in culture density...
following expression of NSP4,18–139 and NSP4,18–175 implying that these variants possess a more potent membrane-
destabilizing activity.

A second assay for membrane-destabilizing activity measured the release of [3H]uridine from the cells. Prior to induction, bacteria were grown in medium containing 5 μCi/ml [3H]uridine. Cells were pelleted, washed twice and then resuspended at the original density in medium lacking [3H]uridine. NSP4 expression was then induced by the addition of 1 mM IPTG and release of radioactivity into the medium used as an indicator of membrane integrity. This assay confirmed the potent membrane-destabilizing activity exhibited by NSP4,18–139 and NSP4,18–175. Synthesis of these variants released ~80–90% of the label within 30 min. A much slower but sustained release of [3H]uridine was observed upon expression of both full-length NSP4 and NSP4,48–91. The remaining proteins examined had little or no apparent effect on the permeability of the bacterial membrane.

The ability of the cytoplasmic tail to enhance the lytic potential of the minimal membrane-permeabilizing region located within residues 48–91 is notable since neither NSP4,46–175 nor NSP4,46–139 affected the stability of the membrane, despite accumulating to much greater levels than any of the other variants. The cytoplasmic tail of NSP4 contains an α-helical coiled-coil domain, located between residues 97–137 (hatched box in Fig. 1), which is proposed to mediate subunit oligomerization (Taylor et al., 1996). Extension of the membrane-destabilizing peptide (NSP4,48–91) at its C terminus to include this coiled-coil domain might facilitate the oligomerization of the resultant polypeptide and thus increase its membrane-destabilizing potential. Oligomerization of the amphipathic sequence might facilitate the formation of a pore or channel in the membrane, similar to other proteins known to alter the permeability of bacterial and eukaryotic membranes (Epand et al., 1995). However the extremely low level of expression of NSP4,18–139 and NSP4,18–175 and the rapid lysis of cells expressing these polypeptides prevented their isolation and subsequent analysis of the oligomeric state.

A 22 amino acid synthetic peptide representing the enterotoxic peptide (residues 114–135) has previously been reported to cause disruption of liposomes and microsomal membrane vesicles (Tian et al., 1996). However, in our experiments, membrane-destabilizing activity could not be attributed to the truncated NSP4 variants that contained the enterotoxic peptide sequence (unless the amphipathic region between residues 48–91 was also included). This difference could be due to different conformations adopted by the 22 amino acid synthetic peptide and the recombinant fragments of NSP4 expressed in this study. However, it should be noted that results derived from the bacterial expression system cannot necessarily be equated with results obtained from liposome permeability studies, particularly when expression of the same 22 amino acid enterotoxic peptide was not directly tested in our experiments. Nevertheless, we have identified a distinct region within NSP4 with the potential to form an amphipathic structure and demonstrated here that recombinant peptides containing this sequence can mediate membrane permeabilization in E. coli.

Localized disruption of the ER membrane might affect the permeability of this organelle. Studies of Ca2+ homeostasis in S9 cells expressing NSP4 support this notion and demonstrate an increase in the rate of Ca2+ leakage from the ER in the presence of thapsigargin, which prevents refilling of the stores (Tian et al., 1995). A similar effect is observed following expression of the coxsackievirus protein 2B in HeLa cells (van Kuppeveld et al., 1997). Analysis of mutant 2B proteins showed that both a region of sequence predicted to form a cationic α-helix and an adjacent hydrophobic domain were critical in mediating this effect. The similar effects that the rotavirus and coxsackievirus proteins have on the permeability of the ER membrane could reflect an underlying similarity in the organization of structural domains. For NSP4, the precise conformation adopted by the membrane-destabilization domain awaits further investigation.

This work was supported by a Project Grant from the Health Research Council of New Zealand. We thank Jan Meyer for critical reading of the manuscript.

References


Membrane destabilization by rotavirus NSP4


Received 10 February 2000; Accepted 18 April 2000