Rotavirus Nonstructural Glycoprotein NSP4 Is Secreted from the Apical Surfaces of Polarized Epithelial Cells[▽]

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NSP4, a nonstructural glycoprotein encoded by rotavirus, is involved in the morphogenesis of virus particles in the endoplasmic reticulum of infected cells. NSP4 is also implicated in the pathophysiology of rotavirus-induced diarrhea by acting as an enterotoxin. To mediate enterotoxic effects in vivo, NSP4 must be secreted or released from rotavirus-infected cells in a soluble form; however, previous studies have indicated that NSP4 is a transmembrane glycoprotein localized within endomembrane compartments in infected cells. In this study, we examined the fate of NSP4 synthesized in Caco-2 cells infected with bovine rotavirus. Our studies reveal that NSP4 is actively secreted into the culture medium, preferentially from the infected-cell apical surface. The secretion of NSP4 is dramatically inhibited by brefeldin A and monensin, suggesting that a Golgi-dependent pathway is involved in release of the protein. In agreement with the proposed involvement of the Golgi apparatus during secretion, secreted NSP4 appears to undergo additional posttranslational modification compared to its cell-associated counterpart and is partially resistant to deglycosylation by endoglycosidase H. Our experiments identify a novel, soluble form of NSP4 secreted from virus-infected cells with the potential to carry out the enterotoxigenic role previously attributed to recombinant forms of the protein.

Infection with rotavirus is the leading cause of viral gastroenteritis in infants and animals worldwide (11). Rotavirus exhibits a marked tropism for polarized cells of the mid and upper epithelium of the small intestine in vivo, resulting in cell death and dysregulation of osmotic homeostasis, leading to profuse watery diarrhea. Various pathophysiological mechanisms have been proposed to account for the clinical symptoms of rotavirus infection, including a reduced surface area of the small intestine, changes in mucosal osmotic permeability following destruction of infected enterocytes, and enhanced electrolyte secretion mediated by a virus-encoded enterotoxin (5, 23, 24).

Most studies of the rotavirus life cycle have used MA104 cells, a poorly differentiated monkey cell line of renal origin. While these cells support virus replication to high titer, they do not exhibit the highly polarized and differentiated phenotype of cells targeted by rotavirus in vivo. Virus replication in MA104 cells involves the assembly of double-layered particles (DLPs) within viroplasm, cytoplasmic inclusions adjacent to the endoplasmic reticulum (ER). DLPs interact with the cytoplasmic domain of NSP4, a viral nonstructural glycoprotein located within the ER membrane that facilitates budding of DLPs into the ER lumen, where the outer capsid proteins VP7 and VP4 are assembled onto the DLP, resulting in infectious virions or triple-layered particles (TLPs) (1, 21). Synchronous infection of MA104 cells is cytopathic, resulting in a rapid loss of cell viability and concomitant release of virions following cell lysis, which occurs progressively within 8 to 16 h of infection (22, 25). In contrast, Caco-2 cells, a well-differentiated polarized cell line of human intestinal origin, remain viable for

several days following infection with rotavirus, during which virions are secreted preferentially from the apical plasma membrane (17, 33). Release of rotavirus from Caco-2 cells involves the association of virus components with lipid microdomains enriched in cholesterol and sphingolipids (8, 30). In these cells, a fraction of VP4 is recruited to cholesterol-rich membranes early in the infectious cycle (30). According to one model, the assembly of VP7 on the surface of the DLP occurs in the ER preceding recruitment of particles into lipid microdomains and the subsequent assembly of VP4 at the cell periphery (9). Studies with polarized epithelial cells have thus revealed a spatial and temporal organization of events in the replication cycle of rotavirus not apparent in MA104 cells. In addition to components of the virion, a fraction of NSP4 is also present within lipid microdomains in rotavirus-infected Caco-2 cells (8, 30). The significance of this association or the fate of this pool of NSP4 is unclear.

NSP4 has also been identified as an enterotoxin capable of causing a phospholipase C-dependent elevation of the intracellular Ca^{2+} concentration when added exogenously to cultured intestinal epithelial cells (10). Several studies have demonstrated the ability of various recombinant and synthetic forms of NSP4, including analogous proteins from non-group A rotaviruses, to induce diarrhea in animal models (2, 10, 15, 16). To perform this enterotoxigenic role in vivo, NSP4 must be released from infected enterocytes in a soluble form capable of diffusing within the intestinal lumen and interacting with the plasma membrane receptors on neighboring epithelial cells. A candidate enterotoxigenic form of the protein was suggested by Zhang et al., who identified a truncated NSP4 peptide (NSP4 112-175) released from rotavirus-infected MA104 cells (40). However, it remains unclear whether this truncated species constitutes an active enterotoxin in vivo or how this molecule might be generated and actively secreted from infected cells.

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The observed differences in rotavirus infection of differentiated versus nondifferentiated cells suggest that intrinsic properties of rotavirus proteins not previously apparent in MA104 cells may be revealed during infection of Caco-2 cells. In this study, we determined the fate of NSP4 synthesized during rotavirus infection of Caco-2 cells. Our results indicate that a significant fraction of NSP4 is secreted from the cellular apical surface prior to the onset of virus-mediated cell death. The secretion of NSP4 was accompanied by a selective posttranslational modification of the protein characteristic of transit through the Golgi apparatus during maturation. Drugs affecting the transport of proteins through the secretory pathway dramatically affected the secretion of NSP4 and revealed an essential role for the Golgi apparatus. The cumulative results indicate that NSP4 and infectious rotavirus particles are released from epithelial cells by distinct routes and offer the prospect that the proposed enterotoxic properties attributed to NSP4 might be mediated by the secreted, soluble form identified here.

MATERIALS AND METHODS

Mammalian cell lines and virus infection. Caco-2 cells were obtained from the American Type Culture Collection and maintained in Dulbecco modified Eagle medium (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum, L-glutamine (2 mM), and penicillin-streptomycin (100 µg/ml) (Sigma, St. Louis, MO). Cells were grown either on plastic multiwell cell culture plates (Nunc) or on 0.4-µm-pore-size polyester semipermeable Transwell filters (Corning). Fourteen-day-postconfluent cell monolayers were infected with trypsin-activated bovine rotavirus (UK strain) at a multiplicity of infection of 10. Cells were washed three times in serum-free Dulbecco modified Eagle medium (DMEM) and incubated in DMEM for 6 h, and the virus inoculum was added for 1 h of incubation, after which cells were maintained in serum-free DMEM for the duration of the experiments.

Western blotting. Culture medium from virus-infected Caco-2 cells was mixed directly with 4× sodium dodecyl sulfate (SDS) sample buffer, electrophoresed on either 12.5% SDS-polyacrylamide or 4 to 12% NuPAGE gels (Invitrogen), and blotted onto nitrocellulose membrane. NSP4 was detected with a rat polyclonal serum raised against purified C90, a polypeptide that contains the carboxy-terminal 90 amino acid residues of NSP4 purified from recombinant *Escherichia coli* (36). Reactive bands were visualized by the addition of horseradish peroxidase-conjugated immunoglobulin G and enhanced-chemiluminescence substrate (Amersham). To analyze cell-associated NSP4, cells were lysed in buffer containing 0.1% TX-100 after removal of the culture medium and centrifuged at 16,000 × g to remove debris, and the supernatant was treated as the medium was.

ELISA. The quantity of NSP4 present in the culture medium and cell lysates was determined by enzyme-linked immunosorbent assay (ELISA) (35). TX-100 (0.1%) was added to medium samples and the purified protein standard to correct for the effect of detergent when comparing the amounts of NSP4 in medium and lysates. Samples (50 to 100 µl) were applied to wells of microtiter dishes (Nunc) and left to adsorb for 1 h. Wells were washed and blocked for 1 h with 0.1% polyvinylpyrrolidone (Sigma). NSP4 was detected by addition of rat anti-C90, followed by peroxidase-conjugated immunoglobulin G and substrate o-phenylenediamine. Rotavirus particles released from infected cells were measured by a sandwich ELISA in which antigen was captured by goat anti-rotavirus serum and bound virus was detected with a rabbit hyperimmune polyclonal serum raised against purified SA11 TLPs.

LDH assays. Cell viability was assessed by measurement of lactate dehydrogenase (LDH) in the culture medium with a Lactate Dehydrogenase (LDH/LD) kit (Sigma). Medium and cell lysates were collected as described above. Portions of each were incubated with 15 nmol pyruvate and 20 µg NAD for 30 min at 37°C. The amount of LDH present in the samples was determined by calibration with a known quantity of L-LDH (Sigma) assayed in tandem with the samples and expressed as a percentage of the total (medium plus lysate) LDH expressed within each monolayer.

Endo-β-N-acetylglucosaminidase H (endo H) digestion. Samples of the medium and cell lysate were incubated in buffer containing 50 mM sodium citrate (pH 5.0)–0.01% SDS for 5 min at 95°C. Samples were allowed to cool, 150 mU of endo H (Roche) was added, and the reaction mixture was incubated at 37°C

for 3 h. Proteins were precipitated with 10% trichloroacetic acid, resuspended in 1× Laemmli sample buffer, and analyzed by Western blotting as described above.

PNGase F digestion. A GlycoProfile II kit (Sigma) was used for deglycosylation with peptide-N-(acetyl-β-glucosaminyl)asparagine amidase (PNGase F). Fractions of medium and cell lysate were incubated for 10 min at 95°C in the presence of 0.1% octyl-β-D-glucopyranoside and 5 mM β-mercaptoethanol. Reaction mixtures were cooled to room temperature and incubated with 2.5 U of PNGase F in 20 mM ammonium bicarbonate buffer (pH 8.3) for 3 h at 37°C. Proteins were precipitated with 10% trichloroacetic acid, resuspended in 1× Laemmli buffer, and analyzed by Western blotting as described above.

RESULTS

NSP4 is secreted from rotavirus-infected Caco-2 cells. Synchronous infection of MA104 cells with rotavirus leads to virus-mediated cytolysis within 12 to 16 h postinfection (hpi). In contrast, rotavirus infection of polarized Caco-2 cells leads to progressive release of virions from the apical surface with only a gradual decline in cell viability. In view of the prolonged viability of rotavirus-infected Caco-2 cells, we sought to determine whether NSP4 was actively secreted into the medium prior to cell death. Cells were infected with bovine rotavirus, and both cell lysates and culture medium were collected at various intervals up to 42 hpi. The samples were subsequently analyzed for the presence of NSP4 by Western blotting. Western blotting revealed the appearance of NSP4 in the cell medium at 16 hpi, which increased progressively up to 42 hpi (Fig. 1A). The identified protein band corresponding to the secreted form of NSP4 exhibited a slightly higher molecular mass and a more diffuse appearance following electrophoresis than the cell-associated form of the protein. To determine whether the NSP4 present in the medium could be due to virus-induced cell lysis, the release of LDH, a soluble cytoplasmic enzyme, was measured during infection. The amount of LDH released from rotavirus-infected cells as a percentage of the total amount of LDH released following detergent lysis varied between 1.7% at 16 hpi and 10.0% at 42 hpi. These results are consistent with previous studies describing the effects of rotavirus on the viability of Caco-2 cells (33). The amount of NSP4 secreted into the medium during this period was quantified with a direct ELISA which was calibrated with purified C90, a recombinant polypeptide that consists of the carboxy-terminal 90 amino acid residues of NSP4 (Fig. 1B). This method revealed that 24.5% of the total NSP4 synthesized was present in the medium by 16 hpi and the amount increased to 35.4% by 40 hpi. The amount of NSP4 present in the medium ranged between 1.5 and 2.5 µg/ml.

Secreted NSP4 is not associated with virions. NSP4 and structural components of virions were previously detected in detergent-resistant microdomains present in rotavirus-infected Caco-2 cells late in the infectious cycle (8, 30). To assess whether the NSP4 released into the medium of infected cells was present in association with virions, we examined the migration pattern of NSP4 after sucrose density gradient centrifugation of the infected culture medium. The migration of rotavirus particles in the gradient was tracked following the addition of 50 µg TLPs (purified independently by CsCl gradient centrifugation) to the infected cell medium and subsequent analysis of the isolated fractions by Western blotting. VP6, a structural protein present in virions, was identified predominantly in gradient fractions 6 to 9 (Fig. 2A). In contrast, NSP4 was present exclusively in fractions 1 to 3 recovered from the top of the gradient (Fig. 2B). Addition of the deter-

FIG. 1. Detection of NSP4 in the medium of rotavirus (RV)-infected Caco-2 cells. (A) Cells grown in multiwell culture dishes were infected with 10 focus-forming units/cell bovine rotavirus (UK strain) at time zero. At various intervals, the medium was recovered from triplicate wells and combined while the residual monolayers were solubilized in an equal volume of buffer containing 0.1% TX-100. Samples were centrifuged in a Microfuge for 15 min, and portions of medium and cell lysate were mixed with sample buffer and analyzed by SDS-polyacrylamide gel electrophoresis and Western blotting. In the blot shown, the portion of medium electrophoresed is fivefold greater than the corresponding amount of cell lysate at each time point to facilitate qualitative analysis of the bands representing the secreted protein. (B) Quantitation of NSP4 in the medium and cell lysate at each time point as assessed by ELISA. (C) Release of LDH from infected and mock-infected Caco-2 cells. P-value <0.05.

gent Triton X-100 (1%) to the medium prior to and during gradient centrifugation did not alter the migration of the protein (Fig. 2C). A similar distribution was observed for a soluble recombinant protein comprising the carboxy-terminal 90 amino acid residues of NSP4 from the bovine rotavirus UK strain (C90) when added to identical sucrose gradients (Fig. 2D). The distribution of infectious rotavirus particles in the gradients following centrifugation of medium, in the absence of exogenous TLPs, was also measured by fluorescent-focus

FIG. 2. NSP4 released into the medium is not associated with rotavirus particles. Medium recovered from cells at 36 hpi was centrifuged through a 5 to 30% linear sucrose gradient for 20 min. Fractions were analyzed for the presence of rotavirus proteins by Western blotting as described in Materials and Methods. (A) Distribution of VP6 present in purified TLPs added to the medium prior to centrifugation by Western blotting with a rabbit polyclonal antiserum raised against purified DLPs. (B) Distribution of NSP4 secreted into the medium from infected cells. (C) Effect of Triton X-100 on the distribution of secreted NSP4 during gradient centrifugation. (D) Distribution of C90, a soluble recombinant fragment of NSP4 that lacks hydrophobic domains. (E) Distribution of infectious rotavirus released into the medium from infected cells measured by fluorescent-focus assay. ffu, focus-forming units.

assay (Fig. 2E). Together, these results indicate that the NSP4 secreted from infected cells is not associated with virions and exhibits a distribution during sucrose gradient velocity centrifugation similar to that of a soluble protein.

The solubility of the secreted NSP4 was further analyzed by differential centrifugation of cell medium. Pellet fractions from a sample of medium centrifuged progressively at $10,000 \times g$, $100,000 \times g$, and $200,000 \times g$ and the final supernatant were collected, and the distribution was analyzed by Western blotting (Fig. 3A). The relative amounts of NSP4 in the pellet and supernatant following the final centrifugation at $200,000 \times g$ were determined by ELISA (Fig. 3B). This experiment revealed that ~80% of the NSP4 secreted by rotavirus-infected Caco-2 cells remained in the supernatant after centrifugation, suggesting that NSP4 is not present within membrane vesicles.

Polarized release of NSP4 from rotavirus-infected cells. Virions are preferentially released from the rotavirus-infected Caco-2 cell apical surface (17). We next examined whether secretion of NSP4 in these cells exhibited a similar polarity.

FIG. 3. Distribution of secreted NSP4 in pellet and supernatant fractions following differential ultracentrifugation. Medium recovered from cells at 36 hpi was ultracentrifuged in an SW65 rotor for 2 h at successively higher speeds, and the distribution of NSP4 was analyzed by Western blotting. (A) P1 to P3 represent equal fractions of the pellets recovered after centrifugation at $10,000 \times g$, $100,000 \times g$, and $200,000 \times g$, respectively. An aliquot of the final supernatant (S/N3) equal to one-fifth of the pellet sample was also analyzed. (B) Relative distribution of secreted NSP4 between the final pellet (P3) and supernatant (S/N3) by ELISA.

Caco-2 cells were seeded into semipermeable filters and maintained for 14 days after reaching confluence. Polarized monolayers were infected with rotavirus, and medium was collected separately from the apical and basolateral chambers at 18 hpi. The presence of NSP4 in each sample was determined by Western blotting and ELISA as described above (Fig. 4). Rotavirus infection of filter-grown Caco-2 cells results in a progressive increase in paracellular diffusion due to disruption of epithelial tight junctions (26). Therefore, to assess the integrity of the epithelial monolayer during the experiment, fluorescein isothiocyanate (FITC)-labeled dextran (4 or 250 kDa) was added to the apical medium at 1 hpi and the paracellular diffusion of this marker during the experimental period was quantified by fluorescence. This experiment revealed that NSP4 was secreted preferentially from the apical surface of the cells with approximately 75% of the secreted NSP4 recovered in this fraction. The paracellular diffusion of FITC-dextran to the basolateral medium ranged from 20% (4 kDa) to 10% (250 kDa). Therefore, it is likely that at least a portion of the NSP4 detected in the basolateral medium had undergone paracellular diffusion following prior release from the apical surface.

Glycosylation status of secreted NSP4. The secretion of NSP4 into the medium of rotavirus-infected Caco-2 cells was associated with an apparent difference in the electrophoretic migration of secreted and cell-associated forms of the protein (Fig. 1A). As previous studies have revealed that NSP4 is glycosylated with high-mannose oligosaccharides on asparagines residues 8 and 18 in the ER, we sought to determine whether the secreted form of the protein was also glycosylated and whether further modification of core oligosaccharide res-

FIG. 4. NSP4 is preferentially secreted from the rotavirus-infected Caco-2 cell apical surface. Filter-grown cells were apically infected with 10 focus-forming units/cell rotavirus. After 1 h, the inoculum was removed and FITC-dextran (FD) with a molecular mass of either 4 or 250 kDa was added to the apical chamber. Histograms show the relative distribution of FITC-dextran, secreted NSP4, and rotavirus particles between the apical and basolateral (b/l) chambers at 18 hpi. The amounts of NSP4 present in equal fractions of medium recovered from the apical and basolateral chambers were compared by Western blotting (inset). Values are means $\pm$ standard deviations of triplicate wells from one of three independent experiments.

idues had occurred during maturation and secretion. Therefore, we compared the sensitivities of the cell-associated and secreted forms of NSP4 to endo H and PNGase F. Endo H cleaves between the *N*-acetylglucosamine residues within the core of *N*-linked high-mannose and hybrid glycans but does not remove complex-type glycans attached in the Golgi apparatus. In contrast, PNGase F cleaves *N*-linked glycan residues at the amino linkage to the asparagine residue. Glycans were readily removed from the NSP4 present in the cell lysate when treated with either enzyme (Fig. 5A). In contrast, most of the secreted NSP4 showed either complete or partial resistance to endo H digestion with the generation of a distinct species with a molecular mass intermediate between those of the fully glycosylated and deglycosylated forms of NSP4 (Fig. 5A, asterisk), although a minor amount migrated similarly to deglycosylated cellular NSP4. The relative amounts of these digestion products did not change following prolonged digestion of secreted NSP4 by endo H for up to 18 h (Fig. 5B). As expected, digestion of cell-associated NSP4 with PNGase F also reduced the molecular mass of the protein to ~ 20 kDa, reflecting the removal of both *N*-linked sugar residues. Digestion of secreted NSP4 with this enzyme also reduced the molecular mass of the protein by a similar amount, but the relative difference in size noted above between the secreted and cellular NSP4 forms remained apparent following digestion (Fig. 4B, lanes 2 and 4). The results of these experiments suggest that most of the *N*-linked glycans present on the secreted NSP4 are of the complex type and have therefore been modified during transit through the Golgi apparatus. In contrast, *N*-linked glycans attached to NSP4 that remains cell associated are susceptible to removal by endo H and are therefore exclusively oligomannose or hybrid in nature, consistent with the observed retention of cell-associated NSP4 within the ER. In addition, the secreted NSP4 appeared to possess an additional modification distinct from *N*-linked glycosylation, as evidenced by its in-

FIG. 5. Secreted NSP4 is resistant to deglycosylation by endo H. (A) NSP4 present in samples of medium (Med) and detergent lysates (Lys) of rotavirus-infected Caco-2 cells was collected at 18 hpi, treated with either endo H or PNGase F for 3 h as described in Materials and Methods, and analyzed by Western blotting. (B) Time course of endo H digestion of NSP4 in medium and lysate. UD, undigested. Time in hours is indicated above some of the lanes. The positions of molecular mass markers (22 and 36 kDa) are shown on the left of each blot.

creased molecular mass relative to the cellular isoform following PNGase F digestion.

Effects of Golgi-disrupting drugs on secretion of NSP4. The progressive release of progeny rotavirus from infected Caco-2 cells has been shown to proceed via a nonconventional secretory pathway that bypasses the Golgi apparatus (17). Given the apparent modification of oligosaccharide residues attached to NSP4 during secretion, we therefore investigated whether secretion of NSP4 might also use a Golgi-independent pathway. To investigate this, we used the drugs brefeldin A, which redistributes *cis* Golgi elements into the ER (18), and monensin, an ionophore that blocks release of secretory vesicles from the *trans* Golgi network. The secretion of NSP4 was significantly reduced by each of these drugs at a concentration that was neither cytotoxic nor reduced the level of NSP4 synthesized in the cells (Fig. 6A). Brefeldin A reduced the amount of NSP4 in the medium at 18 hpi by ~85%, while the effect of monensin was marginally less. In accordance with previous studies, neither drug inhibited the release of rotavirus particles to the medium as previously observed (Fig. 6B). Secretion of NSP4 was not inhibited when infected cells were treated with the microtubule-destabilizing drug nocodazole or cytochalasin D, which disrupts the structure of actin microfilaments.

DISCUSSION

NSP4 is a multifunctional nonstructural glycoprotein with several known properties, including binding to DLPs, the abil-

FIG. 6. Secretion of NSP4 from Caco-2 cells is inhibited by drugs that perturb the function of the Golgi complex. (A) Effects of drugs added 1 hpi on the amount of NSP4 detected in the cell lysate (L) and medium (M) at 18 hpi by Western blotting. As in Fig. 1A, the portion of medium electrophoresed is fivefold greater than the corresponding amount of cell lysate at each time point. (B) Relative amount of NSP4 determined by ELISA in the medium of cells treated with each drug. (C) Relative percentages of particles in medium of infected cells as determined by ELISA. BFA, brefeldin A.

ity to elevate the cytosolic Ca^{2+} concentration, and induction of the unfolded protein response in the ER (1, 21, 34, 39). Recent studies that used RNA interference to selectively silence NSP4 expression in infected MA104 cells have extended the known functions of NSP4, revealing additional roles in the assembly of DLPs and in the transcriptional regulation of viral gene expression (20, 31). The results presented in this paper reveal a novel property of NSP4 synthesized in rotavirus-infected Caco-2 cells. A significant pool of NSP4 is secreted into the cell medium via a Golgi-dependent secretory pathway distinct from the unconventional export route followed by virions.

NSP4 was identified as a transmembrane glycoprotein located in the ER on the basis of (i) protease digestion performed on polypeptide synthesized *in vitro* in the presence of microsomes, (ii) immunofluorescence microscopy, and (iii) enzymatic deglycosylation (3). Recent studies on the subcellular

distribution of NSP4 with fluorescent confocal microscopy in conjunction with cellular markers of endomembrane compartments confirm the presence of the protein in the ER and have identified additional pools of NSP4 in the ER-Golgi intermediate compartment (7, 13, 38). Most recently, NSP4 has been localized in a novel vesicular compartment characterized by the presence of the autophagosomal marker LC3 (4). In polarized epithelial cells, the distribution of NSP4 correlates with that of caveolin-1, a marker of cholesterol-rich membranes and caveolae, flask-like invaginations of the plasma membrane (27). These studies indicate that, to some extent, the distribution of NSP4 may depend on both the context of expression and the cell type. In addition to the spectrum of intracellular locations, a 66-amino-acid proteolytic fragment of NSP4 derived from the cytoplasmic domain has been detected in the medium of rotavirus-infected MA104 cells. This molecule was found to possess enterotoxigenic activity and was secreted via a nonclassical Golgi-independent pathway (40).

The presence of NSP4 in the medium was not due to passive release from dead or dying cells, as the amount of the protein secreted as a proportion of the total amount of NSP4 synthesized greatly exceeded the fraction of LDH present in the medium at each time point. Moreover, treatment of infected cells with Golgi-disrupting agents drastically reduced the amount of secreted NSP4, implying an active process. The release of NSP4 into the culture medium does not appear to involve proteolytic cleavage of membrane-spanning regions, as the secreted NSP4 protein exhibited a slightly greater monomeric molecular mass than the corresponding cellular form when examined by SDS-polyacrylamide gel electrophoresis and Western blotting and the secreted protein remained sensitive to endoglycosidase removal of carbohydrate attached to ER lumen-exposed asparagines residues. Furthermore, our studies show that NSP4 is secreted preferentially from the apical cell surface.

The apparent solubility of secreted NSP4 is somewhat surprising given its transmembrane status. However, certain cellular and viral transmembrane proteins are known to be secreted from mammalian cells without proteolytic removal of hydrophobic membrane anchor sequences. Membrane proteins have been detected in the medium of cultured cells and extracellular fluids *in vivo* within small (50- to 90-nm diameter) membrane vesicles called exosomes (12). These vesicles form by budding into specialized late endosomes referred to as multivesicular bodies and are released into the medium following fusion of the multivesicular bodies and plasma membranes. Several membrane proteins, including MHC class I and II and CD63 have been detected in exosomes secreted from epithelial cells of intestinal origin (37). While we cannot formally exclude the possibility that NSP4 is secreted in an exosome-like membrane vesicle, the distribution of secreted NSP4 following differential ultracentrifugation makes this scenario unlikely as exosomes are sedimented by centrifugation at $100 \times g$ to $150,000 \times g$, unlike NSP4, which remained in the supernatant when centrifuged at $200,000 \times g$. Cellular and viral transmembrane proteins can also be secreted as lipoprotein particles. Hepatitis B virus surface antigen is secreted from infected hepatocytes during chronic hepatitis B and from the apical surface of transfected epithelial cells as a 22-nm-diameter

lipoprotein particle (14, 32). The transmembrane precursor of the secreted lipoprotein is slowly converted into a secreted particulate form that is released into the lumen of the ER and is transported via the Golgi complex to the apical surface of cells. Interestingly, caveolin-1 is also secreted from some cell types as a lipoprotein particle (19). In view of the recent report that caveolin-1 can bind to NSP4, it is possible that NSP4 is secreted from cells in a complex with caveolin-1 or other lipid-binding molecules.

The apparent difference in molecular mass between the secreted and cellular forms of NSP4 evident from Western blot assays is most likely due to a posttranslational modification that occurs within the secretory pathway. The precise nature of this modification is unclear, but the differences in susceptibility to endoglycosidase enzymes are consistent with the modification of glycan residues during transit through the Golgi. Given the inability of PNGase F to completely reduce the molecular mass of secreted NSP4 to that of the cellular form of the protein by removal of N-linked glycan chains, we speculate that the additional mass of the PNGase-treated secreted protein might arise from a further, as-yet-unidentified, posttranslational modification of NSP4 that occurs during secretion. Our inability to detect this modification in the pool of NSP4 that remains associated with the cells suggests that secretion of the protein into the medium occurs rapidly following this modification.

Apical sorting of membrane proteins and secretion of soluble proteins from the polarized-cell apical surface are thought to involve lipid microdomains in the selective transport of cargo from the *trans* Golgi network to the plasma membrane (28, 29). While NSP4 has been detected previously in lipid microdomains isolated from infected Caco-2 cells, it is unclear whether this protein was destined for secretion or in a distinct fraction, possibly associated with virions. In our experiments, secretion of NSP4 was dependent on a functional Golgi apparatus, in contrast to studies that demonstrate Golgi-independent release of virions from Caco-2 cells (17). These data indicate that the pathways followed during release of NSP4 and virions from polarized cells are distinct.

Our study has not addressed the potential physiological properties of the secreted NSP4 protein, which will require its purification from the medium of infected cells. However, the data reported here are clearly consistent with the proposed enterotoxigenic activity of the protein observed previously with various recombinant forms of NSP4. The enterotoxigenic activity of NSP4 was originally attributed to a synthetic peptide comprising residues 114 to 135, but subsequent studies suggested that recombinant forms of the full-length glycoprotein represent a more potent enterotoxin (10). Brunet et al. reported that a molecule secreted from rotavirus-infected Caco-2 cells was able to cause an elevation of intracellular Ca^{2+} when incubated with uninfected cells, consistent with the effects observed with recombinant NSP4 (6). While this study did not identify the molecule responsible for this activity, our present study suggests that the secreted form of NSP4 described here represents a potential candidate.

Our results differ from those presented by Zhang et al., who identified a truncated, nonglycosylated form of NSP4 released from rotavirus-infected MA104 cells infected with SA11 rotavirus by a Golgi-independent process (40). Several factors could ac-

count for these differences. The Caco-2 cells used in these experiments are polarized and well differentiated and do not undergo rapid cytolysis following rotavirus infection. Thus, infected MA104 cells may die prior to entry of NSP4 into the secretory pathway described here or lack cell-specific factors necessary for recruitment of NSP4 into this pathway. Furthermore, in contrast to the relatively small amount of truncated NSP4 recovered from concentrated medium of infected MA104 cells, the NSP4 protein secreted from Caco-2 cells represents a proportionately greater fraction of the total NSP4 synthesized.

In conclusion, the experiments reported in this paper demonstrate that, in addition to its recognized membrane-anchored form within rotavirus-infected cells, NSP4 can exist as an extracellular molecule that is actively secreted from the polarized epithelial cell apical surface. Future studies will be directed at characterizing the nature of the secreted molecule, the molecular determinants of apical secretion, and the functional properties of the secreted protein.

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