Processing of the Rough Endoplasmic Reticulum
Membrane Glycoproteins of Rotavirus SA11

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ABSTRACT The synthesis and oligosaccharide processing of the glycoproteins of SA11 rotavirus in infected Ma104 cells was examined. Rotavirus assembles in the rough endoplasmic reticulum (RER) and encodes two glycoproteins: VP7, a component of the outer viral capsid, and NCVP5, a nonstructural protein. A variety of evidence suggests the molecules are limited to the ER, a location consistent with the high mannose N-linked oligosaccharides modifying these proteins.

VP7 and NCVP5 were shown to be integral membrane proteins. In an in vitro translation system supplemented with dog pancreas microsomes, they remained membrane-associated after high salt treatment and sodium carbonate-mediated release of microsomal contents. In infected cells, the oligosaccharide processing of these molecules proceeded in a time-dependent manner. For VP7, ManαGlCNAc2 and ManαGlCNAc2 were the predominant intracellular species after a 5-min pulse with [3H]mannose and a 90 min chase, while in contrast, trimming of NCVP5 halted at ManαGlCNAc2. VP7 on mature virus was processed to MansGlCNAc2. It is suggested that the α-mannosidase activities responsible for the formation of these structures reside in the ER. In the presence of the energy inhibitor carbonyl cyanide m-chlorophenylhydrazone (CCCP), processing of VP7 and the vesicular stomatitis virus G protein was blocked at ManαGlCNAc2. After a 20-min chase of [3H]mannose-labeled molecules followed by addition of CCCP, trimming of VP7 could continue while processing of G protein remained blocked. Thus, an energy-sensitive translocation step within the ER may mark the divergence of the processing pathways of these glycoproteins.

Membrane glycoproteins locate with a high degree of specificity to a number of subcellular compartments. Due to the complex distribution of proteins normally present in membranes, simple model systems have been sought where the behavior of a few or a single molecular species can be readily defined. To this end, studies of the highly abundant glycoproteins of such membrane maturing viruses as vesicular stomatitis (VSV), sindbis, semliki forest, and influenza have greatly expanded our knowledge of the mechanism of transport to the plasma membrane (reviewed in reference 31). The fine structure of the oligosaccharide moiety serves as a useful marker to indicate the subcellular compartments a specific glycoprotein has traversed. In N-linked glycosylation reactions, a glucose3-mannose9-N-acetylglucosamine2 (Glc3Man9GlcNAc2) core is transferred from a dolichol pyrophosphate carrier to an asparagine residue on the nascent polypeptide chain. This carbohydrate core is extensively trimmed before the addition, in some cases, ofterminal sugars such as N-acetylglucosamine, galactose, fucose, and sialic acid (reviewed in reference 26). While the removal of the outer glucoses and at least one mannose residue has been shown to occur on nascent chains of VSV G protein (4) and thus in the rough endoplasmic reticulum (RER), the location, linkage specificity, and even the total number of the different α1, 2-mannosidases acting during the remaining processing reactions are not clear. Evidence has been presented for both RER (6, 17, 20) and Golgi apparatus (50, 53) activities. The subsequent steps, which include the addition of an N-acetylglucosamine
residue, removal of α1, 3- and α1, 6-mannoses, and transfer of terminal sugars, have been localized to the Golgi complex by a variety of techniques (35, 43, also reviewed in reference 18).

Studies on membrane glycoproteins that are endogenous to the ER are comparatively rare (3, 32, 42), thus a viral model system that displays an ER (or nuclear membrane) site of maturation and shows limitation of its glycoprotein(s) to this organelle could serve as a useful probe in the elucidation of the behavior of these membrane proteins. Rotavirus, a member of the Reoviridae family, is a lytic double-stranded RNA virus that has been shown by electron microscopy to assemble in the RER (1, 25). The simian strain SA11 that grows readily in tissue culture comprises a genome of eleven segments coding for at least twelve polypeptides, five of which are structural and make up the double-layered protein capsid (7, 15). Rotavirus SA11 buds through the RER membrane resulting in the formation of membrane-enveloped particles (1, 48). This membrane is later lost giving rise to the naked capsid, and NCVP5, a 29,000-D protein. Both have been shown to be associated with the ER by immunoelectron microscopy (12, 37, 38).

We have undertaken further studies to establish that VP7 and NCVP5 are integral membrane proteins and to examine the extent of oligosaccharide processing on these molecules. Only high mannose structures were observed on the rotavirus-encoded glycoproteins consistent with their ER location. Carboxyl cyanide m-chlorophenylhydrazone (CCCP) has been reported to block transport of membrane and secretory proteins from the ER (14, 20, 28, 52), and we have tested the effects of this drug on the processing of the rotavirus glycoproteins. VP7 is processed to Man$_9$GlcNAc$_2$ in infected cells, yet in the presence of CCCP, trimming of the carbohydrate moiety is blocked at Man$_5$GlcNAc$_2$. The ramifications of this observation with respect to the transport of the rotavirus glycoproteins are discussed. Portions of this work have appeared previously in abstract form (29, 30).

**MATERIALS AND METHODS**

**Cells and Viruses:** MA104 cells (a fetal rhesus monkey kidney cell line) were provided by Dr. A. R. Bellamy, University of Auckland, Auckland, New Zealand, and grown as monolayers in Dulbecco's modified Eagle's medium (DME, 4 mg/ml glucose) containing 5% calf serum, 5% calf serum, 5% fetal calf serum, 4 mg/ml glucose, 0.4 mM Na$_2$HPO$_4$, 10 mM CaCl$_2$, 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin (all from Gibco Laboratories, Grand Island, NY). Simian rotavirus SA11, also obtained from Dr. A. R. Bellamy, was propagated at a low multiplicity and purified according to the procedure of Street et al. (49). Purified double-shelled particles were dialyzed against Tris-buffered saline, pH 7.4 (TBS; 20 mM Tris-HCl, 140 mM NaCl, 0.4 mM Na$_2$HPO$_4$, 6 mM glucose, 10 mM CaCl$_2$) and stored at −70°C. Triton X-100 mediated lysis of infected cells was assayed by the method of Smith et al. (47). TBS was passed through a hydroxyapatite column (53) and stored at −70°C.

**Carbohydrate Labeling of Infected Cells:** Confluent monolayers of MA104 cells in 35-mm petri dishes were incubated overnight in serum-free DME. The dishes were infected with 30 plaque-forming units (PFU)/cell of SA11 rotavirus that had been preincubated for 30 min at 37°C in TBS containing 10 µg/ml of trypsin (1,250, Difco Laboratories Inc., Detroit, MI) (15°C). Briefly, the cells were infected with 20 PFU/cell of VSV in TBS. After a 1-h adsorption period at 37°C, the cells were washed once with warm TBS and changed to serum-free DME containing 3 µg/ml actinomycin D (act D, Calbiochem-Behring Corp., La Jolla, CA). At 4.5 h postinfection, the medium was changed to glucose-free DME containing 5 µCi/ml [3H]mannose (10-20 Ci/ mmol, New England Nuclear, Boston, MA) and 3 µg/ml act D. At the end of the pulse, the cells were either harvested immediately or chased for varying lengths of time with DME (4 mg/ml glucose) supplemented with 5 mM cold mannose and 3 µg/ml act D. In the experiments involving the drug treatments, stock solutions of CCCP, monensin, or valinomycin (all from Sigma Chemical Co., St. Louis, MO), freshly prepared in 100% ethanol, were added to chase medium containing 1 µCi/ml [3H]mannose (800-1,200 Ci/mmol, Amersham Corp., Arlington Heights, IL). Lysis was induced by washing the cells with cold TBS which were then harvested for SDS PAGE as above.

**Protein Labeling of Infected Cells:** MA104 cells were infected as above with rotavirus. At 4.5 h postinfection, the medium was changed to methionine-deficient DME containing 3 µg/ml act D. 30 min later, the cells were labeled for 60 min with 0.5 µCi/ml [35S]methionine (800-1,200 Ci/mmol, Amersham Corp., Arlington Heights, IL). Lysis was induced by washing the cells with cold TBS which were then harvested for SDS PAGE as above.

**Isolation of Intracellular Virus:** Rotavirus-infected monolayers of MA104 cells in 100-mm petri dishes were pulsed for 5 min, 5 h postinfection, with 4 ml/dish methionine- and glucose-free DME containing 100 µCi/ml [35S]methionine, 100 µCi/ml [3H]mannose, and 3 µg/ml act D, and then washed for 60 min in the presence of DME supplemented with 0.3 mg/ml methionine, 5 mM mannose, and 3 µg/ml act D. The cells were harvested in 10 mM Tris-HCl pH 8.0, 10 mM NaCl, and 1.5 mM MgCl$_2$ and disrupted by douncing. The postnuclear supernatant was extracted with an equal volume of Freon (1, 2, 2, 2-tetrafluoroethane; Eastman Kodak Co., Rochester NY) and the phases separated by centrifugation at 2,000 rpm for 15 min at 4°C. The proteinaceous interface was removed and re-extracted with Freon before being resuspended in sample buffer (100 mM Tris, 5% SDS, 1 mM EDTA, 50 mM dithiothreitol, 10% glycerol, 0.1% SDS, 0.01% bromophenol blue, 100 µg/ml trypsin inhibitor, 200 U/ml Trasylol, 5 mM [35S]methionine acetic acid, 1% benzamidine, and 2 mM PMSF). The aqueous phase was exhaustively re-extracted with Freon and then spun in a Beckman SW41 rotor (Beckman Instruments, Inc, Spinco Division, Palo Alto, CA) at 35,000 rpm for 60 min at 4°C. The resulting pellet which contained viral particles was resuspended in sample buffer before analysis by SDS PAGE.

**Preparation of Oligosaccharides for Column Chromatography:** The [3H]mannose-labeled viral glycoproteins were separated by electrophoresis on preparative 12% SDS polyacrylamide slab gels (5), and treated with 1% SDS polyacrylamide gel electrophoresis (SDS PAGE) following the protocol of Chamberlain (11) or using Amplify (Amenham Corp.). The bands corresponding to VP7 and NCVP5 from the rotavirus-infected cell lysates, or G protein from the VSV-infected cell lysates were excised and treated as previously described (22). Briefly, the gel pieces were swollen in 0.15 M Tris-HCl, pH 8.0, and 10 mM CaCl$_2$, 1 mg pronase (Calbiochem-Behring Corp.) was added, and the samples were incubated under a toluene atmosphere at 37°C for 24 h. Additional pronase was added at 24 and 48 h. The digestion was terminated by boiling the samples, and the gel pieces were removed by centrifugation. The supernatant was lyophilized and resuspended in 0.05 M citrate phosphate buffer, pH 5.0, and the pH was adjusted to 5.5 with 0.2 M citric acid. Endo-β-N-acetylglucosaminidase H (endo H), purified by the method of Tarentino and Maley (51), was added to a final concentration of 0.02 µl/ml [3H]mannose-labeled glycopeptides prepared from Sendai virus-infected chicken embryo fibroblasts (22) and the samples were incubated overnight under toluene at 37°C. The samples were boiled, loaded on a Bio-Gel P-4 column (400 mesh, 1.5 × 157 cm, Bio-Rad Laboratories, Richmond, CA), and eluted with 0.1 M NH$_4$HCO$_3$, 1.1-ml fractions were collected and assayed by scintillation counting.

**Preparation of [3H]Mannose-labeled Rotavirus:** MA104 cells were grown to confluency in 720-cm$^2$ roller bottles and then infected overnight in serum-free DME supplemented with 10% tryptophosphate broth (Dfico Laboratories Inc.), 1X Basal Medium Eagle vitamin solution (Gibco Laboratories), and 0.05 µg/ml ascorbic acid (Mallinkrodt, Inc., St. Louis, MO). The cells were infected at a low multiplicity of infection with 4 PFU/cell of a crude rotavirus stock, and the virus was allowed to adsorb for 60 min at 37°C. At this point, DME containing tryptophosphate broth and 12.5 µg/ml trypsin was added to the cells. 4 h later, the cells were washed once with serum-free DME containing 10 µg/ml of trypsin (1,250, Difco Laboratories Inc.), and the supernatants collected, were adjusted with ammonium bicarbonate to pH 8.0, and the pH was adjusted to 5.5 with 0.2 M citric acid. Endo H was added as described above, and the samples were incubated overnight under toluene at 37°C. The samples were boiled, loaded on a Bio-Gel P-4 column (400 mesh, 1.5 × 175 cm, Bio-Rad Laboratories, Richmond, CA), and eluted with 0.1 M NH$_4$HCO$_3$, 1.1-ml fractions were collected and assayed by scintillation counting.
free DME and changed to 20 ml per bottle glucose-free DME supplemented with glucose at a final concentration of 100 µg/ml, 12.5 µg/ml trypsin, and 25 µCi/ml [3H]mannose. After 3 h, 20 ml per bottle of DME with tryptose phosphate broth and 12.5 µg/ml trypsin was added, and the infection was allowed to proceed to completion. Virus was purified and the oligosaccharides prepared as above, except after pronase digestion, the sample was desalted by Sephadex G-25 chromatography (0.9 × 140 cm, Pharmacia Fine Chemicals, Piscataway, NJ). The oligosaccharides were then separated on a Biogel P-4 column, and the peaks corresponding to ManαGlcNAc, ManβGlcNAc, ManαGlcNAc, and ManβGlcNAc were individually pooled.

Cell-free Protein Synthesis: Rotavirus mRNA was synthesized in an in vitro reaction taking advantage of an endogenous transcriptase in viral cores (33). Rabbit reticulocyte lysate was prepared as described by Pelham and Jackson (36). Dog pancreas microsomal membranes were isolated according to the procedure of Shields and Blobel (46). In vitro protein synthesis was carried out as described (46) with each 25-µl reaction mixture containing 0.02 A260 U of mRNA, 0.125 A250 U of microsomal membranes, 15 µCi of [35S]- methionine, and 5 µg of calf liver tRNA (Boehringer Mannheim Diagnostics, Inc., Indianapolis, IN). The samples were incubated for 90 min at 28°C. For protease protection experiments, 10 µg/ml cycloheximide (Sigma Chemical Co.), 10 mM CaCl2, and 3 mM tetracaine (Sigma Chemical Co.) (45) were then added and the translation mixture incubated at room temperature for 5 min. Triton X-100 was added to one half of the sample at a final concentration of 1% just before the initiation of protein synthesis with 250 µg/ml each of trypsin (Worthington Biochemical Corp., Freehold, NJ) and chymotrypsin (Sigma Chemical Co.). Digestion was allowed to proceed for 60 min at 0°C before termination with 200 U of Trasylol and 1 mM PMSF.

Sodium Carbonate-mediated Release of Microsomal Contents: After translation, the reaction mixture was adjusted to 5 mM CaCl2, and one half was treated with 62.5 µg/ml of each of trypsin and chymotrypsin at 0°C for 60 min. Digestion was terminated by the addition of 200 U of Trasylol and 1 mM PMSF. Both halves of the reaction mixture were then diluted 20-fold with translation salts (20 mM Tris-HCl, pH 7.4, 100 mM KCl, 2 mM CaCl2, 5 mM MgCl2) and loaded into an ultracentrifuge tube over a 1.5 ml high salt sucrose cushion (0.5 M sucrose, 20 mM Tris-HCl, pH 7.4, 500 mM KCl, 2 mM CaCl2, 5 mM MgCl2) and spun in a Beckman SW60 rotor (Beckman Instruments, Inc.) at 40,000 rpm for 30 min at 4°C. The resulting pellets were resuspended in 2.0 ml of 100 mM sodium carbonate, incubated at 0°C for 30 min (19), and then resuspended in the SW60 rotor as above. The supernatants were removed, precipitated with 10% trichloroacetic acid, and processed for SDS PAGE. The pellets from the samples previously treated with proteases were either immediately resuspended in sample buffer or prepared for digestion with endo H. For endo H digestion, the pellet was resuspended in 0.05 M Tris-HCl, pH 6.7, 1% SDS, boiled, and then adjusted to 0.05 M citrate phosphate, pH 5.5, 62 µM of endo H was added, and the mixture was incubated for 60 min at 37°C and then processed for electrophoresis. Similarly, the pellets not originally subjected to proteolysis were either resuspended in sample buffer, or in 10 mM Tris-HCl, pH 7.4, 5 mM CaCl2 before treatment with 250 µg/ml each of trypsin and chymotrypsin for 60 min at 0°C. Human placental lactogen mRNA was a gift from Dr. M. Rosenfeld (NYU School of Medicine).

RESULTS

Rotavirus-specified Proteins

Under the conditions of infection reported here, shutoff of host protein synthesis in MA104 cells infected with rotavirus SA11 was almost complete 4 h postinfection (Fig. 1). Exper-

![Figure 1](image-url)
ments were standardly performed 5 h postinfection where the structural proteins (VP1-VP3, VP6, and VP7) and the nonstructural proteins (NCVP2-NCVP6) were the prominent bands labeled with $^{[35]S}$methionine. The two glycoproteins specified by viral genes 9 (VP7) (8) and 10 (NCVP5) (9) were observed as more diffuse bands of 38 and 29 kD. When infected cells were pulsed with $^{[3]H}$mannose, these were the only two bands appearing on the gel (Fig. 2, lanes 3-5). From the gene sequence, the predicted molecular weight of NCVP5 is actually 19 kD (9), and with the carbohydrate modification (see below) should appear as 23 kD. This aberrant migration on SDS PAGE is entirely due to the N-linked glycosylation, for in the presence of the inhibitor tunicamycin, NCVP5 runs as a 20-kD protein (pNCVP5). Under the same conditions, the unglycosylated form of VP7 (VP7$^u$) was observed as a 35-kD protein (Fig. 2, lane 2).

**In Vitro Translation of Rotavirus mRNA**

Large amounts of rotavirus mRNA can be synthesized in

![Image of gel](https://example.com/gel.png)

**Figure 2** Identification of the rotavirus-specific glycoproteins. Lanes 1 and 6, rotavirus-infected MA104 cells were pulse labeled for 60 min at 5 h postinfection with 40 $\mu$Ci/ml $^{[35]S}$methionine. Lane 2, infected cells were pretreated for 4 h with 2 $\mu$g/ml tunicamycin before labeling with $^{[35]S}$methionine as in lane 1. Lane 3, infected cells were pulse labeled for 60 min with 20 $\mu$Ci/ml $^{[3]H}$mannose. Lane 4, infected cells were pulse labeled for 5 min with 100 $\mu$Ci/ml $^{[3]H}$mannose, and (lane 5) then chased for 60 min in the absence of radioactive precursor. The cells were harvested and the labeled proteins separated by SDS PAGE. VP7$^u$ and pNCVP5 represent the unglycosylated forms of VP7 and NCVP5, respectively.

**Glycosylation on Mature Virus**

Rotavirus is known to assemble in the RER where it remains until cell lysis (25). The extent of processing of the N-linked oligosaccharides modifying VP7 on the mature, purified virus was therefore examined. It was found that processing was extensive with Man$_3$GlcNAc$_2$ and Man$_6$GlcNAc$_2$ predominating, but significant amounts of Man$_3$GlcNAc$_2$ and Man$_6$GlcNAc$_2$ were also present. Consistent with the RER location of the virus and its absence from the Golgi apparatus, complex endo H-resistant glycopeptides...
FIGURE 3. In vitro translation of rotavirus mRNA. (a) Protection of VP7 but not NCVP5 from protease digestion. Total rotavirus mRNA was translated in a rabbit reticulocyte lysate system in the presence of dog pancreas microsomes. Translation mixtures containing microsomes were incubated with 250 μg/ml each of trypsin and chymotrypsin for 60 min at 0°C in the absence (lane 1) or presence (lane 2) of 1% Triton X-100. pVP7 and pNCVP5 refer to the protein precursors of VP7 and NCVP5, respectively. (b) Sodium carbonate-mediated release of microsomal contents. Total rotavirus mRNA was translated in the absence (lane 1) or presence (lane 2) of microsomal membranes. A translation mixture containing microsomes was diluted with translation salts and spun through a high salt sucrose barrier (as outlined in Materials and Methods). The pellet was resuspended in 100 mM sodium carbonate, incubated for 30 min at 0°C, and then respun resulting in a supernatant (lane 3), representing the released microsomal contents and a membrane pellet (lane 4). This experiment was repeated, except that after translation the reaction mixture was incubated with 62.5 μg/ml each of trypsin and chymotrypsin at 0°C for 60 min. Proteolysis was terminated with the addition of 200 U of Trasylol and 1 mM PMSF before the initial pelleting and sodium carbonate treatment (lane 5, microsomal supernatant; lane 6, membrane pellet). To confirm the identity of VP7, a pellet derived as in lane 6 was resuspended in 1% SDS and then digested with 62 mU of endo H for 60 min at 37°C (lane 7). Similarly, to demonstrate that the microsomal vesicles were opened, a pellet treated as in lane 4 was digested with 250 μg/ml each of trypsin and chymotrypsin at 0°C for 60 min before analysis by SDS PAGE (lane 8). The downwardly pointing arrows indicate VP7, while the upwardly pointing arrow marks NCVP5.

FIGURE 4. Biogel P-4 chromatography of mature rotavirus VP7 oligosaccharides. MA104 cells were infected at a low multiplicity of infection with SA11 rotavirus in the presence of trypsin. 5 h post-infection, the cells were changed to glucose-free DME supplemented with 100 μg/ml glucose and 25 μCi/ml [3H]mannose. DME with 10% tryptose phosphate broth was added 3 h later, and the infection was allowed to proceed to completion. Virus was isolated, digested exhaustively with pronase, and the resulting glycopeptides purified by Sephadex G-25 chromatography. [3H]Mannose-labeled glycopeptides from sindbis virus-infected cells were added, and the high mannose oligosaccharides were released by digestion with endo H and separated on a Biogel P-4 column. The arrows mark the peak elution fractions of the sindbis virus markers with S1–S3 representing complex endo H-resistant glycopeptides and M2N–

were not observed on the Biogel P-4 column (Fig. 4). Extensive digestion of the peaks corresponding to ManαGlcNAc–ManαGlcNAc with jack bean α-mannosidase resulted in free mannose and an oligosaccharide migrating with the mobility of ManGlcNAc (data not shown), confirming that the oligosaccharides contained terminal mannose residues.

Processing of Intracellular VP7 and NCVP5

Rotavirus-infected cells were pulsed for 5 min with [3H]mannose and chased for varying amounts of time in the absence of radioactive precursor. There was no evidence for secretion of the virally encoded glycoproteins into the chase medium as determined by SDS PAGE or by kinetic analysis of trichloroaetic acid-precipitable material in the medium. Similarly, no extensive intracellular degradation could be detected (data not shown). Intracellular-labeled proteins were separated by SDS PAGE, and the bands corresponding to VP7 excised from the gels and oligosaccharides prepared for Biogel P-4 chromatography. As with mature virus VP7, complex oligosaccharides were not observed on total intracellular VP7 at any chase time (Fig. 5, a–f). Processing to ManαGlcNAc was evident after 5 min of chase and to M2N, the high mannose oligosaccharides of composition mannoses/N-acetylglucosamine–mannoses/N-acetylglucosamine. 
Trimming also occurred on the oligosaccharide chains of the nonstructural glycoprotein NCVP5 (Fig. 7, a–e). However, unlike VP7, processing of NCVP5 was slower with Man$_6$GlcNAc after a 20-min chase. By 90 min of chase, Man$_9$GlcNAc and Man$_6$GlcNAc were the predominant species, and further processing did not occur, even after extensive chase periods (data not shown). This suggests that the trimming reactions are highly regulated and not simply a result of the time of exposure of the substrate to the appropriate enzymes.

Since both the membrane- and virus-associated VP7 were presumably present at this stage of infection, it was important to assess the relative contributions of each to the overall processing pattern seen in Fig. 5. This was accomplished by taking advantage of the behavior of viral particles upon extraction of the infected cell lysate with Freon. Rotavirus-infected cells were pulsed for 5 min with $[^3]$H]mannose and $[^{35}]$S)methionine, and then chased for 60 min. After treatment of the homogenized cells with Freon, viral particles could be cleanly pelleted from the aqueous phase as assayed by SDS PAGE (data not shown), while the unassembled VP7 molecules resided in the aqueous–organic phase interface. Analysis of the oligosaccharides present on these two forms of the protein showed that the membrane VP7 (Fig. 6a) had a processing profile similar to that of total intracellular VP7 at the same time of chase (Fig. 5e), while the carbohydrates modifying virus VP7 were not as extensively trimmed as demonstrated by the much reduced amounts of Man$_6$GlcNAc (Fig. 6b). This suggests that the majority of the VP7 pool at this time in infection is membrane bound and that processing of the oligosaccharide chain to the final form found on the mature virus (Fig. 4) can occur on the intracellular virus particle.

Trimming also occurred on the oligosaccharide chains of the nonstructural glycoprotein NCVP5 (Fig. 7, a–e). However, unlike VP7, processing of NCVP5 was slower with Glc$_5$Man$_9$GlcNAc, Glc$_6$Man$_9$GlcNAc, and Man$_6$GlcNAc, the major species, after a 5-min pulse with $[^3]$H]mannose and a 5-min chase. Due to the small size of the protein moiety of

**Figure 5** Biogel P-4 chromatography demonstrating processing of VP7 oligosaccharides in rotavirus-infected cells. Rotavirus-infected cells were pulsed for 5 min with 100 µCi/ml $[^3]$H]mannose and chased for 5–90 min (a–l) before harvesting. The viral glycoproteins were separated by SDS PAGE and the bands corresponding to VP7 excised, rehydrated, and digested extensively with pronase. The resulting glycopeptides were mixed with $[^{14}]$C)mannose-labeled sindbis virus-infected cell glycopeptides, digested with endo H, and then analyzed by Biogel P-4 chromatography. The arrows mark the peaks of elution of the sindbis virus markers with S1–S3 representing complex endo H–resistant glycopeptides and M$s$N–M$s$N, the high mannose oligosaccharides of composition mannoses$\alpha$-acetylglucosamine$_2$–mannose$_3$N-acetylglucosamine$_2$.

**Figure 6** Biogel P-4 chromatography comparing processing on the unassembled and virus-associated forms of VP7. Rotavirus-infected cells were pulsed for 5 min with 100 µCi/ml each of $[^3]$H]mannose and $[^{35}]$S)methionine and then chased for 60 min before harvesting. The postnuclear supernatant was extracted with Freon resulting in a proteinaceous interface and an aqueous phase, from which assembled viral particles were pelleted. The interface (a) and virus pellet (b) were analyzed by SDS PAGE; the bands corresponding to VP7 were excised and oligosaccharides prepared for chromatography as in Fig. 5.
secretory proteins from the ER to the Golgi apparatus (14, 20, 28, 52). To ascertain whether the amount of α1,2-mannosidase processing observed on the rotavirus glycoproteins was due to such transport, infected cells were pulsed for 5 min with \(^{3}H\)mannose, chased for 60 min in the presence of 100 μM CCCP, and the glycopeptides of VP7 prepared and digested with endo H. Analysis by Biogel P-4 chromatography (Fig. 8b) showed that processing was blocked at Man\(_6\)GlcnNAc. The drug had no effect on the trimming of the NCVP5 oligosaccharides which normally remain at Man\(_6\)GlcnNAc (data not shown). As a control to demonstrate that transport to the Golgi apparatus was indeed inhibited, the experiment was repeated with VSV-infected Mal04 cells. VSV is a plasma membrane maturing virus containing a single glycoprotein G, whose carbohydrate is N-linked and complex (40). The action of α-mannosidase II and the terminal glycosylating enzymes present in the Golgi complex (18, 35, 43) confers upon the oligosaccharide chains resistance to endo H. The resulting glycopeptides elute earlier from a P-4 column (Fig. 9a). However, inclusion of CCCP in the chase medium prevented the appearance of complex oligosaccharides and blocked processing at Man\(_6\)GlcnNAc (Fig. 9b), analogous to VP7.

CCCP is thought to block between the ER and Golgi complex, a supposition difficult to reconcile with the extent of processing of VP7 and its probable ER location. A comparison of the VP7 oligosaccharide profile obtained in the presence of CCCP (Fig. 8b) with the time-dependent processing patterns shown in Fig. 5 (notably that in Fig. 5c) suggests that the drug-induced block may occur by 20 min after synthesis. If that were the case, and if the protein were allowed

![Figure 7 Biogel P-4 chromatography demonstrating processing of NCVP5 oligosaccharides in rotavirus-infected cells.](image_url)

NCVP5, processing intermediates seemed to separate out on SDS PAGE accounting for the double band seen on gels (for example, Fig. 2, lane 3). For a 10-min chase period, the upper band corresponded to the glucose containing oligosaccharides, while the lower band was mainly Man\(_6\)GlcNAc (Fig. 7, c and d). The final processing pattern, consisting of Man\(_6\)GlcNAc with some Man

![Figure 8 Biogel P-4 chromatographic analysis of oligosaccharides derived from rotavirus VP7 chased in the absence or presence of CCCP.](image_url)

**Oligosaccharide Processing in the Presence of CCCP**

CCCP is an uncoupler of oxidative phosphorylation and has been shown to prevent movement of membrane and

FIGURE 7 Biogel P-4 chromatography demonstrating processing of NCVP5 oligosaccharides in rotavirus-infected cells. Rotavirus-infected cells were pulsed for 5 min with 100 μCi/ml \(^{3}H\)mannose and chased for 5-60 min (a-e). The viral glycoproteins were separated by SDS PAGE and the bands corresponding to NCVP5 excised and prepared for chromatography as described in Fig. 5. At the chase times where a double band was observed such as 10 min, the upper and lower portions of the band were processed independently (c and d).

FIGURE 8 Biogel P-4 chromatographic analysis of oligosaccharides derived from rotavirus VP7 chased in the absence or presence of CCCP. Rotavirus-infected cells were pulsed for 5 min with 100 μCi/ml \(^{3}H\)mannose and then chased for 60 min in the absence (a) or presence (b) of 100 μM CCCP. The oligosaccharides from VP7 were prepared for chromatography as described in Fig. 5.
to progress just beyond the block, processing might continue normally. To test this possibility, rotavirus-infected cells were pulsed for 5 min with [3H]mannose and chased for 20 min before treatment with 100 μM CCCP for an additional 60 min. Under these conditions, substantial processing occurred (Fig. 10, a and b), generating a final pattern similar to that of the unassembled form (Fig. 6). We have shown (Fig. 3b) that VP7 is also membrane associated since it remained membrane bound after high salt treatment and release of the microsomal contents at alkaline pH by sodium carbonate. The exact orientation of the molecule is not clear, though it may be anchored in the membrane by the second hydrophobic sequence of 17-20 amino acids at the NH2-terminus, each of which is sufficiently long to form a membrane spanning region. Repeated NH2-terminal hydrophobic sequences are also a feature of another ER glycoprotein, HMG-CoA reductase (13). In the case of VP7, the first segment appears to be a signal sequence since most or all of it is removed posttranslationally (16). In contrast, NCVP5 is highly unusual in that its two potential glycosylation sites are present in the first hydrophobic sequence at asparagines 8 and 18. By the criteria of partial endo H digestion (2, 16), both sites are glycosylated. NCVP5 was susceptible to protease digestion after in vitro translation in the presence of dog pancreas microsomes (Fig. 3a, lane 1) under conditions in which VP7 was protected. Since N-linked glycosylation occurs co-translationally at the luminal aspect of the RER, NCVP5 is a transmembrane protein.

We have shown (Fig. 3b) that VP7 is also membrane associated since it remained membrane bound after high salt treatment and release of the microsomal contents at alkaline pH by sodium carbonate. The exact orientation of the molecule is not clear, though it may be anchored in the membrane by the second hydrophobic sequence of 17-20 amino acids at the NH2-terminus, resulting in an extremely short cytoplasmic domain. Our data suggest that VP7 transfers from the membrane of the immature virus in the RER lumen to form the outer shell of the mature virion. To our knowledge, no other animal virus exhibits this behavior, the closest parallel being the filamentous phage as exemplified by M13, whose major coat protein is initially an integral membrane protein, yet upon virus budding comprises the phage outer coat which lacks a membrane (54).

Our observations on the processing of the intracellular VP7 pertain almost entirely to the membrane-bound form of the molecule. Virus-associated VP7 displayed an oligosaccharide profile different from that of the unassembled form (Fig. 6). The latter was virtually identical to the total intracellular VP7 under the same pulse-chase conditions, implying that the virus overall energy effect on the cells, since increasing the concentration of glucose in the chase medium served to mitigate the action of CCCP (14). While 50 μM CCCP added to chase medium containing 4 mg/ml glucose failed to block glycoprotein processing, it was totally effective when present in medium containing 0.1 mg/ml glucose (data not shown). The concentration of CCCP used in these experiments was not cytotoxic because protein synthesis restarted by 15 min after removal of the drug (data not shown).
Figure 10. Biogel P-4 chromatography demonstrating processing of oligosaccharides derived from VP7 or G protein in the presence of CCCP. Rotavirus-(a and b) or VSV-(c and d) infected cells were pulsed for 5 min with 100 μCi/ml [3H]mannose and either chased for 20 min and harvested (a and c) or chased for an additional 60 min in the presence of 100 μM CCCP before preparation for SDS PAGE. Oligosaccharides from rotavirus VP7 or VSV G protein were processed for chromatography as described in Fig. 5.

VP7 pool is a relatively insignificant part of the total at this time of infection.

The extensive α1,2-mannosidase processing of the VP7 high mannose oligosaccharides could be accounted for by either the molecule reaching the Golgi apparatus, or by the presence of the enzymes within the ER. We do not think the former possibility likely because VP7 is not secreted from infected cells, degraded intracellularly, or observed to accumulate in the Golgi apparatus by the immunofluorescence assay noted above. Recycling of ER molecules, such as unassembled VP7 through the Golgi apparatus where further α-mannosidase trimming could take place, has also been proposed (44), though there has never been compelling evidence for this concept. However, since the amount of high mannose trimming on VP7 isolated from intracellular virus (Fig. 6b) after a short pulse and chase is less than that found on virus after essentially a long pulse (Fig. 4), processing must occur on the virus particle in order to obtain the final pattern. It seems doubtful that VP7 in mature virus would recycle through the Golgi apparatus because virus particles are not seen there, or in any other organelle except the RER and its extension, the nuclear envelope (39; Poruchynsky, M., and P. Atkinson, manuscript in preparation). In addition, we have recently shown (Poruchynsky, M., C. Tyndall, G. Both, F. Sato, A. R. Bellamy, and P. Atkinson, manuscript submitted for publication) that the products of specific deletion mutants of VP7, transfected into COS 7 cells under the control of the late SV40 promoter, are secreted and that the glycosylation becomes resistant to endo H. This demonstrates that if VP7 traverses the Golgi apparatus it can become terminally glycosylated. Complex forms of VP7 are never observed in infected cells, making it highly unlikely that VP7 reaches the Golgi apparatus. Our results therefore argue for the existence of α1,2-mannosidase activities in the ER supplemental to that partially purified from a rat liver RER fraction (6), or the yeast RER α-mannosidase active on invertase which removes only one α1,2-mannose at a specific site (10, 17, 27). Postulation of additional activities would in any event seem necessary because their products have been observed in the processing of several endogenous ER membrane glycoproteins, such as ribophorin I in cultured rat liver hepatocytes, which is processed to Man3GlcNAc2 (42), and HMG-CoA reductase which is trimmed to Man3GlcNAc2 (32). Furthermore, the microsomal forms of other secreted glycoproteins have been shown to contain processed high mannose oligosaccharide chains (23, 24). Other proteins, such as sindbis virus PE2 (22) and rat liver transferrin (34), clearly can be processed by α1,2-mannosidases in the Golgi apparatus, and thus the protein backbone may influence where these reactions occur in the cell.

The effect of CCCP on the processing of rotavirus VP7 oligosaccharides is particularly interesting since it blocked trimming at Man4GlcNAc2. CCCP is an uncoupler of oxidative phosphorylation and a proton transporter which collapses...
the membrane potential and pH gradient. Recent evidence shows that the RER displays both of these features (41), however, specific alteration of either of these seems unlikely to be responsible for the inhibition, since agents that affect each parameter independently have no effect on VP7 processing. The drug may therefore act through lowering the energy level in the cells. Since α-mannosidase processing reactions are not energy dependent, the block may be in the movement of VP7 to the subcompartment containing the appropriate enzymes. NCVP5, whose processing halts at Man$_5$GlcNAc$_2$ (Fig. 7), may not be transported to this compartment. This is supported by the observation that VP7 pulse-labeled for 5 min with $[^{3}H]$mannose, chased 20 min to pass the energy barrier marked by formation of Man$_5$GlcNAc$_2$ (cf. Fig. 8), then further chased in the presence of CCCP were trimmed almost normally to the Man$_5$GlcNAc$_2$/Man$_3$GlcNAc pattern typical of the untreated molecule (Fig. 10b). VSV G protein by contrast, under the same conditions, does not show such continued extensive processing (Fig. 10d; cf. Fig. 9a). These results suggest a divergence of the VP7 and G protein processing pathways, perhaps mediated in an energy-sensitive step. Since membrane-bound VP7 was the predominant form of the molecule in the infected cell at this time of infection (Fig. 6), it is clear the block in processing of labeled VP7 oligosaccharides was not due to a possible inhibition by CCCP of the initial virus budding into the RER. Similarly, the fact that in the presence of the drug trimming continued on VP7, and that VSV G protein was transported through the Golgi apparatus (Fig. 10), argues that the drug was not immediately cytotoxic. In summmation, though we do not rule out that VP7 could recycle back to the ER through the Golgi apparatus, we think a more likely explanation is that an energy barrier exists somewhere within the ER which limits the presentation of the Man$_5$GlcNAc$_2$ form of VP7 to at least the Man$_4$GlcNAc$_2$-generating α1,2-mannosidase.

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47. Smith, E. M., M. K. Estes, D. Y. Graham, and C. P. Gerba. 1979. A plaque assay for the simian rotavirus SA11. J. Gen. Virol. 43:513-519.
48. Soler, C., C. Musalem, M. Lorono, and R. T. Espejo. 1982. Association of viral particles and viral proteins with membranes in SA11-infected cells. J. Gen. Virol. 44:983-992.
49. Street, J. E., M. C. Cossar, W. F. Chadderton, and A. R. Bellamy. 1982. Sequence diversity of human rotavirus strains investigated by Northern blot hybridization analysis. J. Virol. 43:369-378.
50. Tabas, I., and S. Kornfeld. 1979. Purification and characterization of a rat liver Golgi α-mannosidase capable of processing asparagine-linked oligosaccharides. J. Biol. Chem. 254:11655-11663.
51. Tarentino, A. L., and F. Maley. 1974. Purification and properties of an endo-α-N-acetylgalactosaminidase from Streptomyces griseus. J. Biol. Chem. 249:811-817.
52. Tartakoff, A. M., P. Vassalli, and M. Detraz. 1977. Plasma cell immunoglobulin secretion: arrest is accompanied by alterations of the Golgi complex. J. Exp. Med. 146:1332-1345.
53. Tulisani, D. R. P., S. C. Hubbard, P. W. Robbins, and G. Touster. 1982. α-1-Mannosidases of rat liver Golgi membranes: mannosidase II is the GlcNAc-Mans-cleaving enzyme in glycoprotein biosynthesis and mannosidase IA and IB are the enzymes converting Mans precursors to Man9 intermediates. J. Biol. Chem. 257:3360-3368.
54. Webster, R. E., and J. S. Cashman. 1978. Morphogenesis of the filamentous single-stranded DNA phages. In The Single-Stranded DNA Phages. D. T. Denhardt, D. Dressier, and D. S. Ray, editors. Cold Spring Harbor Laboratory, New York. 557-569.