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Rotaviruses Code for Two Types of Glycoprotein Precursors

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Rotaviruses are nonenveloped viruses that code for two glycoproteins: a structural glycoprotein (VP7) and a nonstructural glycoprotein (NS29). The precursor to VP7 (37K) was shown to contain a 1.5K cleavable signal sequence. The 37K precursor was authentically processed (signal sequence cleaved and the polypeptide "core" glycosylated) when synthesized in a cell-free system supplemented with dog pancreatic microsomes. Similar experiments were performed with the nonstructural glycoprotein precursor (20K); however, the 20K precursor contained an integral (noncleavable) signal sequence. Both precursors were inserted into membranes cotranslationally and both glycosylated products underwent post-translational oligosaccharide processing. The results suggest a morphogenetic scheme for the simian rotavirus SA11.

Key words: dog pancreatic microsomes, signal sequences, rotavirus glycoproteins

Many viral systems have provided excellent models of glycoprotein biosynthesis and processing [13,16]. Biochemical characterization of the rotaviruses has shown that their infectious form does not contain an envelope, yet they contain a major N-linked structural glycoprotein of the high mannose type [1,8,9,11], reminiscent of viruses that are surrounded by a lipid envelope.

Rotaviruses are unusual because the mature virion is unenveloped, yet during morphogenesis, the particles bud through the rough endoplasmic reticulum (RER) and transiently obtain an envelope (Fig. 1). The role of the envelope is unknown, but it is not required for infectivity [12]. The envelope is normally lost within the RER lumen, but enveloped particles accumulate when infected cells are treated with the glycosylation inhibitor tunicamycin [7,25]. Thus, although rotaviruses are the only known nonenveloped viruses containing a major structural N-linked glycoprotein, this is not altogether unexpected, considering the similarities in morphogenesis with the enveloped viruses.

Another unusual feature of rotaviruses is that they also code for a nonstructural glycoprotein. Nonstructural glycoproteins are rare among all virus groups and the

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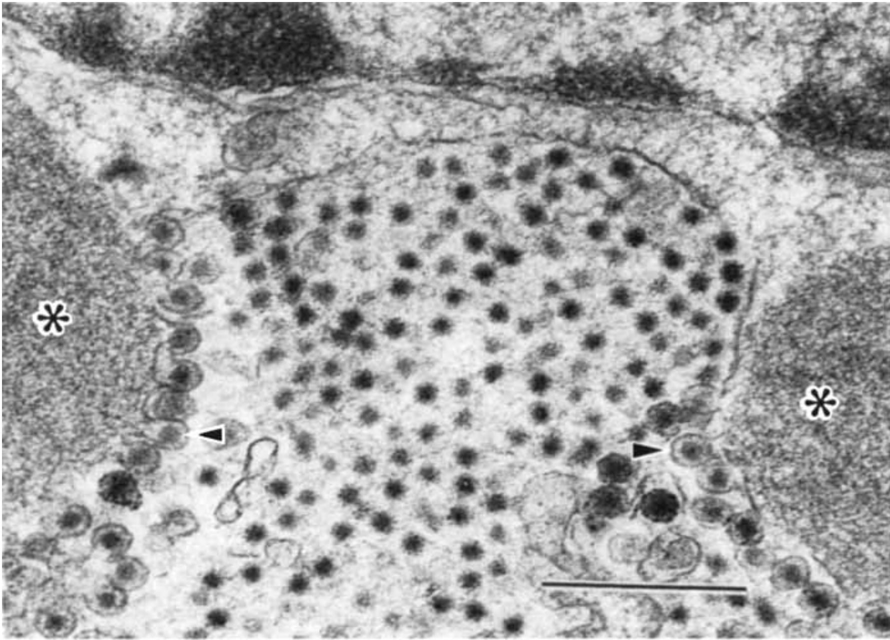

Fig. 1. Electron micrograph of SA11 infected cell (14 hr postinfection [p.i.]) showing cytoplasmic inclusion bodies or viroplasms (asterisks) from which immature viral particles bud (arrowheads) into the cisternae of the endoplasmic reticulum, becoming enveloped in the process. Mature particles in the interior of the cisternae have lost the envelope. Bar = 0.5 μm.

rotaviruses and adenoviruses are the only nonenveloped viruses known to produce a high mannose type of nonstructural glycoprotein (Table I). Current evidence suggests that this nonstructural glycoprotein is involved in the budding process [25].

We have studied the synthesis of the rotavirus glycoproteins both in vivo and in vitro. We found that the structural glycoprotein precursor contains a cleavable signal sequence, whereas the nonstructural glycoprotein precursor has an integral signal sequence. The topology of the two glycoproteins with respect to the RER membrane was also investigated and we discuss the implications of these data with respect to viral morphogenesis.

MATERIALS AND METHODS

Cells and Virus

Simian virus SA11 was plaque-purified three times, propagated at low multiplicity (<0.1 plaque-forming units [PFU]/cell) and assayed in MA104 cells [10,20]. VP7, the SA11 structural glycoprotein, exhibits structural polymorphism [11]. We used clone 3, a strain producing a 38K glycosylated VP7, and clone 28, a strain producing a 35.5K nonglycosylated form of VP7 for these experiments.

Radiolabeling of Intracellular Rotavirus-Coded Polypeptides

Confluent monolayers of MA104 cells were infected with trypsin-activated SA11 virus at a multiplicity of 20–30 PFU/cell and labeled with [^{35}S]-methionine (40 μCi/ml; 1,200 Ci/mmol; Amersham Corp., Arlington Heights, IL) in either the

TABLE I. Glycoproteins of Nonenveloped Animal Viruses

Virus	Nucleic acid[a]	Budding[b]	Glycoprotein(s)				
			Structural[c]	Nonstructural[c]	Type[d]	MW[e]	Reference
Adeno	dsDNA	—	Fiber	—	O	61K	[14]
				E3	N-hM	25K	[17]
Papova	dsDNA	—	—	T-Agn	?	94K	[30]
Parvo	ssDNA	—	—	—	—	—	
Picorna	ssRNA	—	—	—	—	—	
Reo	dsRNA-s	—	ulc	—	O	72K	[18]
Orbi	dsRNA-s	ER/PM	—	—	—	—	
Rota	dsRNA-s	ER	VP7		N-hM	38-36K	[1,8,9]
				NS28	N-hM	29K	[1,8,9]

[a]dsDNA, double-stranded deoxyribonucleic acid; ssDNA, single stranded deoxyribonucleic acid; ssRNA, single-stranded ribonucleic acid; dsRNA, s-segmented double-stranded ribonucleic acid.
[b]ER, endoplasmic reticulum; PM, plasma membrane.
[c]Nomenclature of individual virus family glycoproteins (see [13,16] for review).
[d]Type of linkage of oligosaccharide moiety. O-serine or threonine-linked; N-asparagine-linked; hm-high mannose.
[e]Molecular weight in thousands.

presence or absence of tunicamycin (1.0 μg/ml; Calbiochem-Behring, San Diego, CA) [8]. Infected cells were harvested in 100 μl of RIPA buffer (150 mM NaCl, 1% sodium desoxycholate, 1% Triton X-100, 0.1% sodium dodecyl sulphate [SDS], 10 mM Tris-hydrochloride (pH 7.2), and 1% Trasylol; FBA Pharmaceuticals, New York). The resulting lysate was centrifuged in a Beckman Airfuge at 100,000g for 60 min, and the supernatant fractions used for further analyses.

Preparation of Dog Pancreatic Microsomal Membranes

Dog pancreatic microsomal membranes were prepared by the method of Shields and Blobel [31] and Scheele et al [28], and stored at −70°C in 50% glycerol until used. They retained activity for >2 yr.

In Vitro Transcription and Translation of Rotavirus mRNA

Rotavirus mRNA was prepared using the endogenous viral polymerase [20]. Transcripts from all 11 genes or mRNA species 10, fractionated and recovered from acid-urea agarose gels [21], were translated using the procedure of Pelham and Jackson [24] as modified by Mason et al [20]. The translation system was supplemented with RNAsin™ (2,000 U/ml, Biotech, Madison, WI) and microsomal membranes (3–6 A_{260}U/ml).

Protease Digestion of the Cell-Free Translation Reactions

Polypeptides synthesized in vitro in the presence of microsomal membranes were treated with trypsin (100 μg/ml final concentration) in the presence of tetracaine-HCl (Sigma, final concentratrion, 3mM) [4,5,29] either with or without Triton X-100 (1% final concentration) [4,5,29]. The polypeptides in the treated samples were precipitated with 80% acetone, dried under vacuum, and resuspended in 50 μl of sample buffer (5 mM Tris-HCl, pH 6.8, 8% glycerol, 1% SDS, 0.5 M urea, 5% 2-mercaptoethanol, and 0.003% phenol red). They were boiled for 2 min immediately before electrophoresis.

SDS-Page

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using 12% polyacrylamide slab gels (0.75 mm thick) and the Laemmli discontinuous buffer system containing 0.5 M urea [19,20]. The gels were prepared for fluorography using Autofluor (National Diagnostics, Somerville, NJ) and exposed to x-ray film (Kodak X-Omat, XAR-5) at −70°C.

Immunoprecipitation of Polypeptides From Infected Cells and Rabbit Reticulocyte Lysates

Monospecific antiserum to the VP7 glycoprotein was prepared by immunizing guinea pigs with dansylated VP7 that was purified from polyacrylamide gels. Infected cell lysates and rabbit reticulocyte reaction mixtures were prepared in RIPA buffer, and the immunoprecipitation reactions were performed using Kessler's procedure [15] as modified by Ericson et al [8].

Endo H Digestion of Infected Cell and Cell-Free Samples

Polypeptides from infected-cell and cell-free samples were digested with Endo H (Miles Biochemicals; Elkhart, IN) as described [9].

RESULTS

A Cleavable Signal Peptide Is Present on the 37K Precursor to VP7

Cell-free translation systems have often been needed to demonstrate the presence of a signal peptide on glycoprotein precursors since such moieties are only transiently present in cells. Supplementation of the cell-free system with microsomal membranes reconstitutes the rough endoplasmic reticulum in vitro and allows polypeptide precursors to be authentically processed.

Translation of clone 28 rotavirus mRNA (nonglycosylated VP7 phenotype) in the absence of microsomal membranes (Fig. 2, lane A) resulted in the production of a 37K polypeptide that was not detected in infected cells (lane C). When the translation was performed in the presence of membranes, the amount of 37K polypeptide was greatly decreased with the concomitant appearance of a polypeptide migrating at 35.5K (lane B). Peptide mapping and immunoprecipitation using monospecific antiserum against VP7 (data not shown) confirmed the precursor-product relationship of the 37K and 35.5K polypeptides and demonstrated that the 35.5K polypeptides made in vivo and in vitro were identical. The polypeptide that migrates just ahead of the 35K nonstructural polypeptide (lane A, *) is most likely due to endogenous protease activity in rabbit reticulocyte lysates acting on the 37K polypeptide [22], since translations performed in wheat germ systems (data not shown) did not produce this polypeptide.

Translation of clone 3 mRNA (glycosylated VP7 phenotype) in the absence of membranes (lane G) also yielded a 37K polypeptide that was not detectable in clone 3 infected cells (lane E). Addition of membranes to the translation mixture produced a 38K polypeptide (lane F), instead of the 37K polypeptide, suggesting a precursor-product relationship of the 37K and 38K polypeptides analogous to that of the 37K and 35.5K polypeptides described above. Immunoprecipitation of the in vitro translation products and of the infected cell lysates with anti-VP7 serum (lanes H-K) and

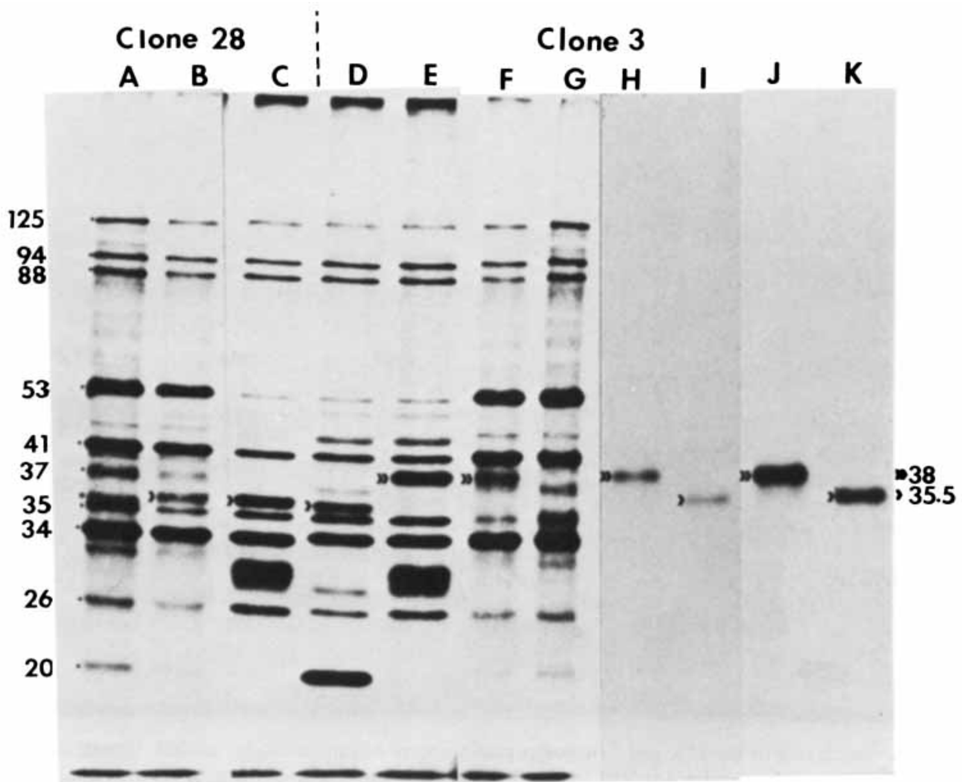

Fig. 2. The 37K polypeptide contains a signal sequence. Polypeptides synthesized in vitro or infected cells were labeled with [^{35}S]-methionine and analyzed by SDS-PAGE as described in Materials and Methods. The estimated molecular weights ($\times 10^3$) of the primary gene products are shown and the virus-specific polypeptides which have been described previously [8] are indicated by a dot. Lanes A and B. Cell-free translation of clone 28 mRNA in rabbit reticulocyte lysates without or with microsomal membranes, respectively. Lane C. 10-min pulse of clone 28-infected cells at 4 hr p.i. Lanes D and E. 10-min pulse of clone 3-infected cells (4 hr p.i.) in the presence or absence of TM, respectively. Lanes F and G. Cell-free translation of clone 3 mRNA with or without microsomal membranes, respectively. Lanes H–K. Immunoprecipitation of the 38K polypeptide synthesized in vitro (H and I) or from clone 3-infected cells (J and K) with anti-VP7 serum. The immunocomplexes were incubated with 0.1 M KH_2PO_4 (H and J) or Endo H (I and K), as described in Materials and Methods. The single arrow (>) denotes the 35.5K polypeptides, and the double arrows (> >) denote the 38K polypeptides. The asterisk in lane A indicates a 35K polypeptide occasionally made without microsomes in rabbit reticulocyte lysates which is not made in cell-free systems derived from wheat germ (see text) (from Ericson et al [9], with permission).

treatment of the immunoprecipitates with either Endo H (Lanes I and K) or KH_2PO_4 (lanes H and J) showed that both the in vitro (Lanes H and I) and the in vivo (lanes J and K) synthesized 38K polypeptides were glycosylated and were immunologically related. Peptide mapping (data not shown) confirmed the relationships between the 37K and 38K polypeptides described above and a 35.5K polypeptide produced in infected cells treated with the glycosylation inhibitor tunicamycin (lane D). Since

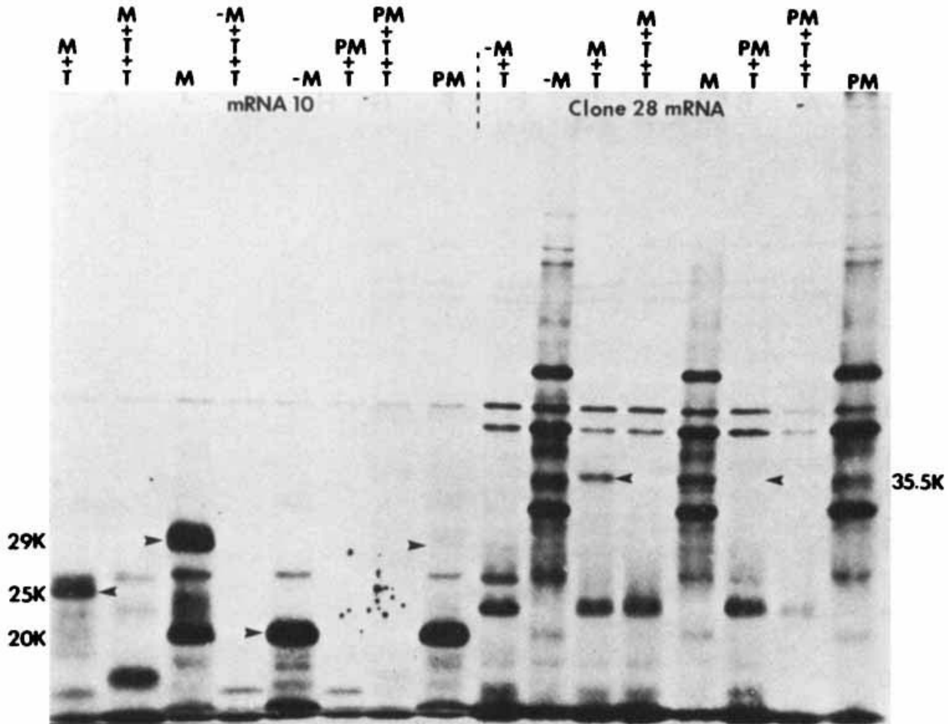

Fig. 3. Processing of the 20K and 37K polypeptides occurs cotranslationally. mRNA 10 (see text) or clone 28 mRNA were translated at 30°C in the reticulocyte lysate system in the presence (M) or absence (−M) of dog pancreatic microsomal membranes for 60 min. Alternatively, at the end of the 60-min translation period (without microsomes), cycloheximide was added (100 μg/ml) along with 6 A260 U/ml microsomal membranes (PM), and incubation was continued at 30°C for an additional 30 min. The translation reactions were divided and incubated with (T) or without 100 μg/ml TPCK-treated trypsin in the presence (T + T) or absence of 1% Triton X-100 detergent. Lefthand side. Arrows (▶) indicate the fully glycosylated (29K) polypeptide, the partial digestion product (25K), and the nonglycosylated precursor (20K). Note the absence of the 29K in the PM lane. Righthand side. Arrows (▶) indicate the 35.5K polypeptide (M+T). Note the absence of the 35.5K polypeptide in the PM+T and PM lanes.

removal of the high mannose oligosaccharide from the 38K polypeptide with Endo H treatment produced the 35.5K polypeptide, the clone 3 37K polypeptide, like the clone 28 37K polypeptide, has a signal sequence.

The Nonstructural 29K Glycoprotein Contains a Noncleavable Signal Sequence

When unfractionated mRNA was used to program the cell-free system in the presence of microsomes, the nonstructural glycoprotein (29K) was not synthesized, although the synthesis of its 20K precursor polypeptide decreased (Fig. 2, lanes B, F). It remains unclear why the 20K nonstructural polypeptide is not glycosylated by the microsomal membranes when unfractionated mRNA is used. However, the 29K glycoprotein was produced when purified mRNA 10 which codes for the 20K polypeptide [21] was used (Fig. 3).

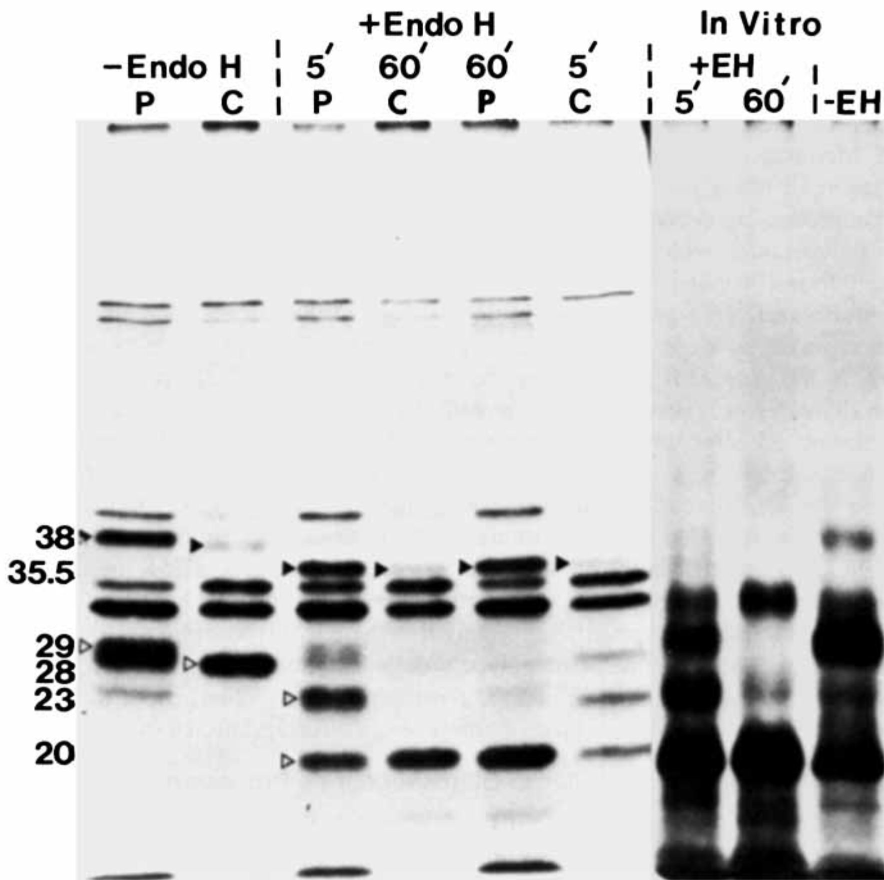

Fig. 4. Oligosaccharide processing of the SA11 glycoproteins. MA104 cells were infected and pulse-labeled with [^{35}S]-methionine for 10 min (P) at 4 hr p.i. and either harvested immediately or chased with excess unlabeled methionine for 2 hr (C) and then harvested as described in Materials and Methods. The resultant infected cell lysates (left six lanes) were then incubated with (+ Endo H) or without (− Endo H) β-N-acetylglucosaminidase H (Endo H, 3 μg/ml) for 5 min or 60 min as indicated in the figure. The solid black arrows (▶) refer to the 38K (− Endo H) or 35.5K (+ Endo H) polypeptides. The open arrows (▷) refer to the 29K and 28K polypeptides (− Endo H) and to the 23K and 20K polypeptides (+ Endo H). The right three lanes represent the cell-free synthesis of the 29K polypeptide using purified mRNA 10 followed by treatment with Endo H (+EH) for 5 min or 60 min or no Endo H Treatment (− EH). Note the conversion of the 29K polypeptide to the 23K and 20K polypeptides with Endo H treatment (from Ericson et al [9], with permission).

Endo H digestion of the 29K polypeptide synthesized in vitro confirmed that this polypeptide was glycosylated. Complete Endo H digestion yielded a product of 20K, and the comigration of the 20K polypeptide after Endo H digestion (Fig. 4) with the 20K primary gene product suggests that the 20K polypeptide contains an integral (noncleavable) signal sequence. This conclusion is also supported by the comigration of the 20K from tunicamycin-treated infected cells and the 20K synthesized in vitro (Fig. 2).

Processing of the SA11 Glycoproteins Occurs Cotranslationally

To determine if insertion of the 37K and 20K polypeptides into membranes can occur posttranslationally, we translated clone 28 mRNA or purified mRNA 10 in the absence of microsomes for 60 min and inhibited further translation with cycloheximide. Membranes were then added and incubation continued for 30 min at 30°C. No processing of either the 37K or 20K polypeptides was observed (Fig. 3), suggesting that the processing events occur cotranslationally.

Polypeptides which contain a signal peptide are usually inserted into membranes. To test whether the 20K or 35.5K glycoprotein precursors were inserted into membranes, mRNA was translated in the presence of microsomes and aliquots of the reaction mixtures were treated with trypsin, either in the presence or absence of Triton X-100 detergent (to dissolve the membrane). The 35.5K polypeptide from clone 28 mRNA was protected from trypsin digestion in the absence of detergent and was completely digested in the presence of Triton, suggesting that resistance to protease digestion was supplied by the membrane barrier. The 37K precursor polypeptide was also digested by trypsin both when membranes were absent and when membranes were added posttranslationally. An identical experiment performed with the 29K glycoprotein showed that in the absence of detergent a 25K fragment was protected from trypsin digestion. In the presence of detergent, the 29K glycoprotein was digested to a 16K fragment, suggesting that the 29K glycopeptide is a transmembrane protein. The 20K precursor polypeptide was digested in the absence of membranes or when membranes were added posttranslationally. Thus, both the 20K and 37K polypeptides were inserted into membranes as a cotranslational event.

Rotavirus Glycoproteins Undergo Oligosaccharide Processing

We have previously shown that the 29K and 38K glycoproteins undergo posttranslational trimming. In addition, the trimming of the 29K glycoprotein can be inhibited by canavanine (an arginine analog) [8]. To determine the mechanism of this processing, Endo H digestions of pulse-labeled and pulse-chased rotavirus glycopeptides were performed (Fig. 4). The deglycosylated digestion products (35.5K and 20K) of the pulse-labeled samples comigrated with the digestion products from the chase samples, suggesting that the trimming occurred in the oligosaccharide moieties and not in the polypeptide backbone. Partial Endo H digests were also used to determine the number of glycosylation sites on each of the glycoproteins. The partial digests of the 29K glycoprotein revealed a band migrating at 23K as well as the 20K band, suggesting that 29K glycoprotein contained at least two glycosylation sites. In contrast, the partial Endo H digest of VP7 only produced the 35.5K precursor, demonstrating this glycoprotein has only one glycosylation site.

DISCUSSION

The presence of signal sequences on a polypeptide precursor implies (and directs) a need for that polypeptide to be sorted. The fact that the rotavirus VP7 precursor (37K) has a signal sequence suggests that this protein must become associated with the rough endoplasmic reticulum. This association has been confirmed by immunocytochemical methods using anti-VP7 serum [26] and by cellular fractionation studies [32]. The oligosaccharide moiety of VP7 was trimmed following the initial glycosylation event, suggesting that Golgi membranes may be involved in the sorting

of VP7 as mannosidases are known to reside in the Golgi apparatus [27,33]. The characterization of VP7 as a glycoprotein containing only N-linked high mannose oligosaccharides offers this as a new model to study the biosynthesis of high mannose glycoproteins.

The gene 10 primary product (20K) contains an uncleaved signal sequence. Therefore, the 29K glycoprotein is another animal virus glycoprotein (in addition to the p62 polypeptide of Sindbis virus [2], and the neuraminidase of influenza virus [3]) that contains an integral signal sequence. The significance of polypeptides containing integral signal sequences is not yet understood. However, our data demonstrating that the 29K glycoprotein is partially accessible to trypsin digestion after insertion into membranes is consistent with this being a transmembrane protein. All other polypeptides containing integral signal sequences (except ovalbumin [23]) are also transmembrane proteins.

Both glycoproteins probably play critical roles in the morphogenesis of the outer capsid of rotavirus particles. The membrane sequestered state of VP7 suggests that this protein may be assembled into the outer protein shell of the virus particle as a consequence of the "subviral" particle budding through the RER membrane. Indeed, both immature enveloped particles and mature nonenveloped double-capsid particles within the RER lumen contain VP7 [Petrie, unpublished observation].

The insertion of the nonstructural glycoprotein into intracellular membranes invites speculation concerning its potential role in rotaviral morphogenesis. We propose several hypotheses for the function of this nonstructural glycoprotein. First, the 29K glycoprotein may act as a "receptor" that mediates budding of the "subviral" particles into the RER. In this role, this glycoprotein would be transiently associated with immature SA11 virus particles, consistent with its designation as a nonstructural polypeptide. If NS29 is a transmembrane protein, this would also be consistent with this protein acting as a receptor for "subviral" particles.

Second, the 29K glycoprotein may act as an inhibitor of the transport of glycoproteins out of the RER and Golgi membranes. Since rotaviruses bud exclusively into intracellular membranes and not at the plasma membrane, VP7 and NS29 are probably not transported to the cell surface; however, this needs to be examined directly for NS29. No viral structural antigens can be detected on the infected cell surface by antisera to purified virus particles [26].

Third, NS29 may act as a calcium ion "pump." This would be expected to enhance the assembly process of the outer capsid layer, as Ca^{+2} is required for the integrity of the double-shelled particles [6]. It is anticipated that the expression of the cloned gene 10 in eukaryotic cells will allow direct testing of these hypotheses and thus determine the role of the nonstructural glycoprotein for rotavirus replication.

Why two different types of signal sequences (cleavable and noncleavable) would be encoded by the rotaviruses is unclear. One possibility, alluded to above, is that VP7 and NS29 need to be "compartmentalized" in the same subcellular locations, but in different orientations with respect to the membrane, implying that the function(s) of these polypeptides is dependent upon secondary, tertiary, and perhaps quarternary structural constraints. It is interesting to note that some 20K glycoprotein precursor can be detected in the infected cell (without tunicamycin treatment) [8] and in the cell-free system when membranes are present throughout synthesis. In contrast, the VP7 precursor (37K) has never been detected in the infected cell and is present in much lesser amounts than is the 20K in the cell-free system supplemented with

membranes. This suggests that "competition" may occur between the two glycoprotein precursors, perhaps mediated by differential affinity of signal recognition particle [34] for one signal sequence over the other.

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