Sequences in Rotavirus Glycoprotein VP7 That Mediate Delayed Translocation and Retention of the Protein in The Endoplasmic Reticulum

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Abstract. Glycosylation and translocation of the simian rotavirus protein VP7, a resident ER protein, does not occur co-translationally in vivo. In pulse-chase experiments in COS cells, nonglycosylated VP7 was still detectable after a 25-min chase period, although the single glycosylation site was only 18 residues beyond the signal peptide cleavage site. After labeling, glycosylated and nonglycosylated VP7 was recovered in microsomes but the latter was sensitive to trypsin (i.e., the nascent protein became membrane associated) but most of it entered the ER posttranslationally because of a rate-limiting step early in translocation. In contrast with the simian protein, bovine VP7 was glycosylated and translocated rapidly. Thus, delayed translocation per se was not required for retention of VP7 in the ER. By constructing hybrid proteins, it was further shown that the signal peptide together with residues 64–111 of the simian protein caused delayed translocation. The same sequences were also necessary and sufficient for retention of simian VP7 in the ER. The data are consistent with the idea that certain proteins are inserted into the ER membrane in a loop configuration.

ROTAVIRUSES, as members of the family *Reoviridae*, have a segmented double-stranded (ds)[1] RNA genome and replicate in the cytoplasm of infected cells. Electron microscopy has shown that viral morphogenesis begins in the cytoplasm in viroplasmic inclusions where the central core and single-shelled particles are assembled (for review see references 4 and 13). The latter enter the ER during a budding process that is mediated by the nonstructural, resident ER glycoprotein NS28 (3, 4). The outer capsid layer of mature virus particles consists of VP4 and the glycoprotein VP7 (14), but it is not clear how these are acquired. An inspection of VP4 sequences (7, 20) suggests that the protein has no signal peptide and presumably enters the ER as part of the budding particle. However, VP7 contains a cleavable signal peptide that directs it to the ER independently of the single-shelled particles (12, 29, 32). Analysis of the carbohydrate attached to VP7 shows that it is of the high-mannose type (18) indicating that the protein is retained in the ER where virus maturation occurs. This has been confirmed by the expression of cloned VP7 genes in COS cells (23, 28). Other studies have also shown that VP7 is retained in the ER as an integral membrane protein (18).

The rotavirus VP7 signal peptide (referred to as H2; reference 32) is involved in directing the protein to the ER and retaining it there. This was shown by replacing the H2 signal sequence with the signal peptide from influenza virus hemagglutinin, such that cleavage still occurred at the normal site; VP7 produced from this hybrid precursor was secreted (28). Thus, the H2 signal peptide is unusual in that it has two functions. As this work progressed, it was also observed that glycosylation of VP7 destined for the ER appeared to occur much more slowly than glycosylation of its secreted counterpart. This was unexpected because most proteins are glycosylated co-translationally during entry into the ER (2, 5, 25). Since the folding of some proteins and their transportation from the ER can be greatly affected by glycosylation (24, 31), we considered the possibility that the observations made with VP7 might be connected with the mechanism by which this protein is retained in the ER. In this work, we examine the kinetics of VP7 glycosylation in more detail and investigate the reason for its delay. We also identify sequences in VP7 that, together with the signal peptide, modulate entry of VP7 into the ER and are necessary and sufficient to retain it there.

Materials and Methods

Mutation and Construction of VP7 Genes

The construction of rotavirus genes H2VP7 and HAFVP7 has been described (28). H2VP7 is derived from codons 30–326 of the open reading frame of SA11 gene 9 and is sufficient to code for the major viral outer capsid protein (29). HAFVP7 carries the signal peptide of influenza hemagglutinin attached to Phe47 of SA11 VP7 (Fig. 1). The cloning of the VP7 gene from a bovine rotavirus (strain NCDV) has previously been described (17). The NCDV VP7 gene was excised from pBR322 as a Dra I–Pst I fragment

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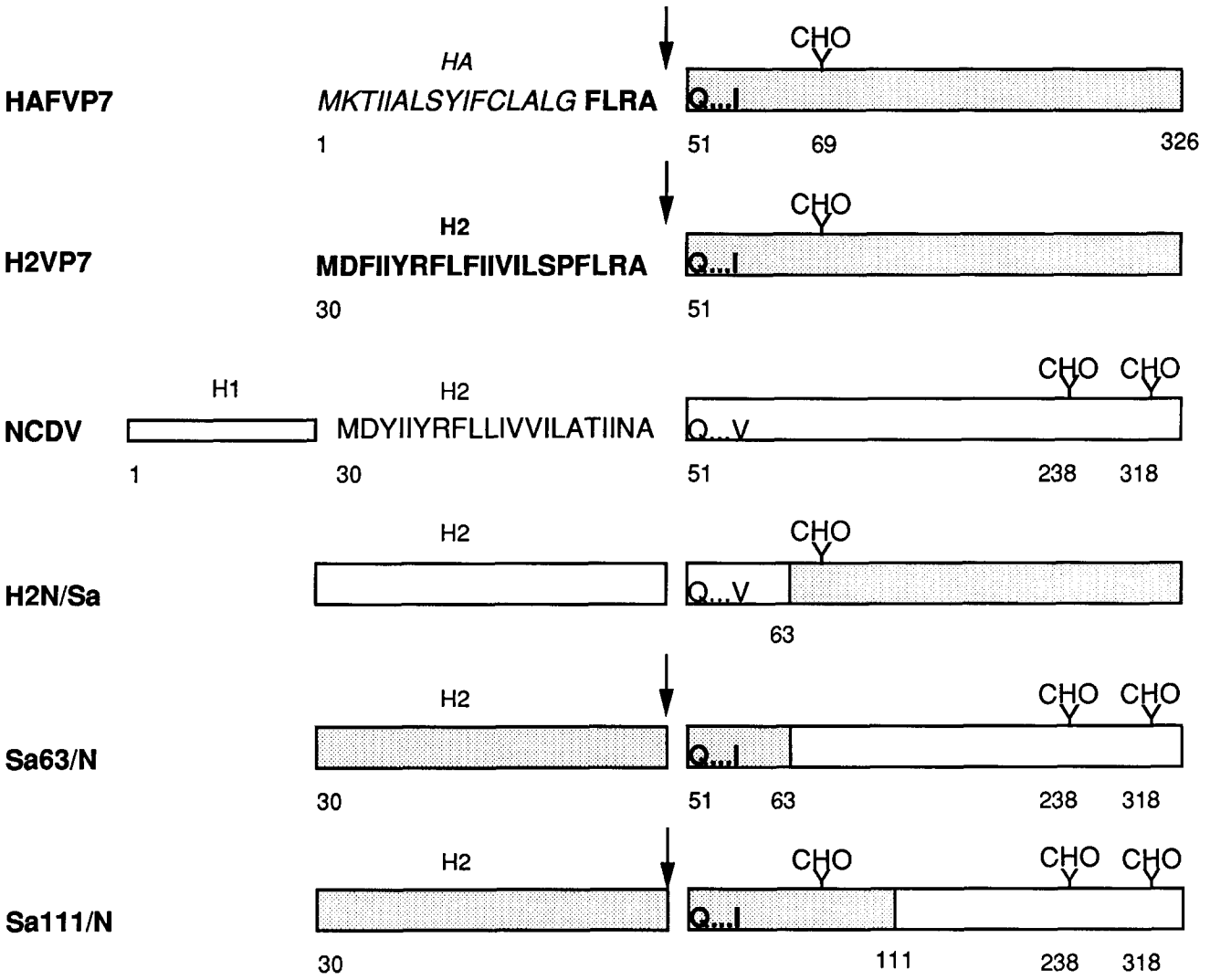

Figure 1. Structure of wild-type and modified VP7 proteins. Residues from the simian (strain SA11) protein H2VP7 (wild type) are shown in bold type and hatched tones and numbered according to the open reading frame (8). Influenza HA residues are in italics; bovine rotavirus (strain NCDV; wild type) VP7 residues are in plain type and open boxes. *CHO* indicates mannose-rich, N-linked glycosylation sites at Asn residues 69, 238, or 318. The numbers 63 and 111 indicate residues at which simian and bovine hybrid VP7 proteins are fused. Known signal peptide cleavage sites are indicated by arrows.

of ~1-kb, Bam HI linkers were blunt-end-ligated to the Dra I end, the gene was recut with Bam HI and was subcloned into Bam HI/Pst I-cut plasmid pBCB06 (9). The gene was prepared for subcloning into an SV40 vector pJC119 as follows. Recombinant pBCB06 was cut with Hind III, and the linear DNA was made blunt-ended with T4 DNA polymerase. Xho I linkers were ligated to the DNA and a ~1-kb fragment containing the gene was prepared by digestion with Bam HI and Xho I. This fragment was incorporated in the correct orientation in the SV40 vector by ligation with Eco RI/Bam HI and Eco RI/Xho I fragments of 4.2 and 2.8 kb, respectively, which had been prepared from pJC119 (32) (NCDV, Fig. 1). An Nco I site was introduced into the NCDV gene by subcloning the Bam HI–Pst I piece from pBCB06 into Bluescribe M13+ (Stratagene, San Diego, CA). Single-strand DNA was rescued using helper phage M13K07 and an Nco I site was made by introducing a silent base change at position 234 using a mutagenesis kit (Bio-Rad Laboratories, Richmond, CA) (28). Using sites in the polylinker of the plasmid carrying the mutated gene, a 5′ Bam HI/Sph I 3′ fragment was prepared, and this was ligated into the Bam HI–cut SV40 vector in the presence of a Bam HI–Sph I adaptor. Hybrid molecules containing NH_2 terminal sequences from simian VP7 attached to downstream sequences of the bovine protein were constructed using unique Nco I and Bal I restriction sites in the genes. Xho I–Nco I and Xho I–Bal I fragments of 150 and 295 bp that encoded residues 30–63 and 30–111 of simian (SA11) VP7, respectively, were inserted into the SV40 vector in place of the equivalent bovine (NCDV) sequences by three fragment ligation (32) to create Sa63/N and Sa111/N, respectively (Fig. 1).

Construction of VP7-S Antigen Fusion Proteins

The malaria S antigen gene (in the Eco RI site of Bluescript M13$^+$KS; Stratagene), from which the internal Bam HI site was removed (28), was cut with Nde I and made blunt-ended by end filling with DNA polymerase (Klenow fragment). The DNA was phenol extracted and precipitated with ethanol, cut with Hind III to remove the 5′ sequences of the gene and purified by agarose gel electrophoresis. The H2VP7 gene, subcloned as an Xho I fragment (23) into the Sal I site of Bluescribe M13$^+$, was cut with Hind III and Bal I and a ~300-bp fragment containing the 5′ sequences of H2VP7 was purified by gel electrophoresis. This was ligated with the Hind III–Nde I (blunt) DNA from above to create the gene H2-111/S. This was excised from the Bluescript plasmid with (5′) Xho I and Bam HI (3′) and subcloned into the SV40 vector pJC119, as described for H2S (28). The gene HA-111/S was constructed as follows. A 5′ Nde I (blunt)–Bam HI 3′ fragment of ~750 bp was prepared from the Bluescript plasmid H2-111/S. A (5′) Xho I–Bal I (3′) fragment of ~250 bp was prepared from a plasmid containing the gene HAFVP7 (28), and this was ligated with the above S antigen fragment and plasmid pSV_L (Pharmacia Fine Chemicals, Uppsala, Sweden), which had been cut with Xho I and Bam HI.

The integrity of the gene constructions was checked by restriction digestion or sequence analysis.

Transfection of DNA and Detection of Expressed Proteins

DNA was introduced into COS cells by electroporation and proteins expressed were digested as required with endo H and analyzed by immunoprecipitation, gel electrophoresis, and autoradiography as previously described (28).

Preparation of Microsomes

A crude preparation of microsomes was made from [^{35}S]methionine-labeled, transfected COS cells essentially as described (34). The cells were washed twice with PBS, harvested, and pelleted at 1,000 *g* for 5 min, then resuspended in 2 ml of hypotonic buffer (1 mM $MgCl_2$, 10 mM Tris HCl, pH 7.5), and allowed to swell on ice for 5 min before Dounce homogenization. The disrupted cells were incubated for 1 h in the presence of trypsin, trypsin plus 1% NP-40, or without protease at 0 or 37°C, as indicated in the appropriate figure legend. The reactions were stopped by the addition of aprotinin (100 μg/ml) and 10 mM PMSF (in isopropanol). The samples were then pelleted through 10% sucrose (10 ml) in an SW41 rotor (Beckman Instruments, Inc., Palo Alto, CA) at 38K rpm for 5 h at 4°C (16). The pellets were dissolved in a solution containing 1% NP-40, 0.4% DOC, 66 mM EDTA, and 10 mM Tris (pH 7.4), and centrifuged to remove nuclei and debris. VP7 was recovered from the supernatants by immunoprecipitation and analyzed by gel electrophoresis.

Results

Kinetics of Glycosylation of Simian H2VP7

Genes H2VP7 and HAFVP7 code for precursor proteins with different signal peptides that are rapidly cleaved to yield the same, mature, simian VP7 (Fig. 1) (28). However, it was consistently observed that, after a short pulse-labeling period, only one species of HAFVP7 was present in cells compared with two H2VP7-derived proteins. The H2VP7-derived proteins were therefore examined in more detail to ascertain whether there was a product/precursor relationship between them. COS cells transfected with H2VP7 or HAFVP7 genes were pulse-labeled with [^{35}S]methionine and then chased for various times. VP7 was recovered by immunoprecipitation, and some samples were digested with endo H. For HAFVP7 only one product was detected, even at zero time chase (Fig. 2 *A*, lane *1*), and this was endo H-sensitive (Fig. 2 *A*, lane *5*), and therefore glycosylated. Two H2VP7-derived proteins were visible after labeling (Fig. 2 *B*, lane *1*). The larger was endo H-sensitive (Fig. 2 *B*, lane *6*), and therefore glycosylated. The lower band was endo H-resistant and not glycosylated. This product appeared to chase into the upper band with a half-time of 16 min (Fig. 2 *B* and Fig. 3). Thus, despite being rapidly processed, the different signal peptides in H2VP7 and HAFVP7 affected the behavior of mature VP7 in vivo both with respect to its ability to locate in the ER (28) and to its rate of glycosylation.

Translocation of Simian H2VP7 Is Delayed

Glycosylation in vivo can occur on nascent polypeptide chains; i.e., cotranslationally (2, 5, 25). Glycosylation of secreted HAFVP7 therefore occurred as expected; its unglycosylated precursor was not detectable in pulse-labeled cells (Fig. 2 *A*). However, glycosylation of H2VP7 occurred slowly. Since signal peptide cleavage occurs on the lumenal

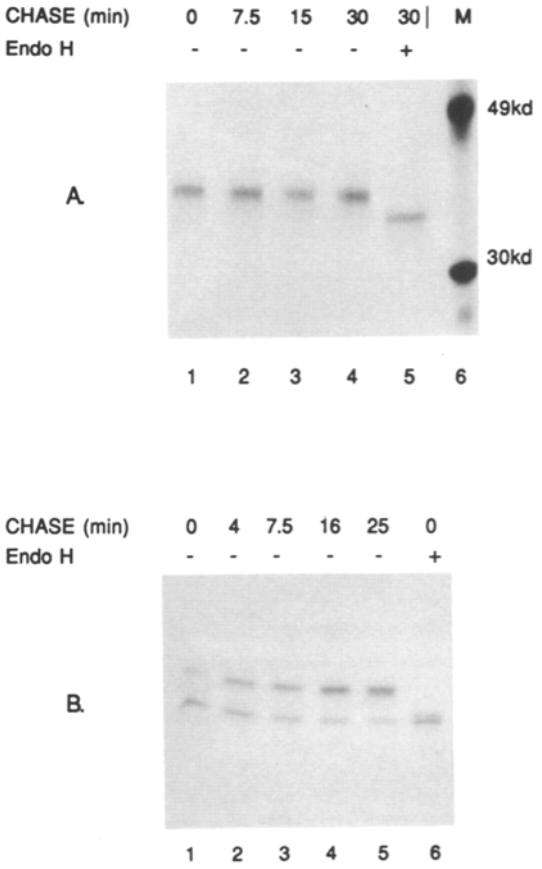

Figure 2. Differential rate of glycosylation of HAFVP7 and H2VP7 proteins. COS cells were transfected with (*A*) HAFVP7 and (*B*) H2VP7 genes, pulse-labeled with [^{35}S]methionine for 15 min and chased with unlabeled methionine (25 mM) for the times indicated. VP7 was recovered by immunoprecipitation, and the samples indicated were digested with endo H before analysis by gel electrophoresis and autoradiography.

side of the ER membrane (at Gln51) and the single N-linked glycosylation site in H2VP7 lies only 18 residues downstream at Asn69 (Fig. 1), this suggested that a rate limiting step occurred at an early stage of simian VP7 translocation. Alternatively, the protein may have been glycosylated slowly in the ER due to conformational constraints. To distinguish between these possibilities, the trypsin sensitivity of microsome-associated H2VP7 and HAFVP7 polypeptides was examined.

COS cells were transfected with these genes and pulse-labeled for 10 min. Microsomes were then prepared and incubated with or without trypsin. In control experiments when the microsomes were first disrupted by detergent, all proteins recovered were sensitive to digestion with trypsin (Fig. 4 *A*, lane *4*, and *B*, lane *5*). In the absence of detergent, the single, glycosylated HAFVP7 protein (*open arrowhead*) was trypsin resistant, showing that it had been completely translocated into the ER (Fig. 4 *A*, lanes *2* and *3*). Of the H2VP7 polypeptides detected after pulse-labeling (Fig. 4 *B*, lane *2*, *open arrowheads*), the glycosylated (*upper*) protein was resistant to digestion with trypsin (although it migrated as a slightly broader band after digestion; *solid arrowheads*), but the lower species was completely sensitive to the enzyme

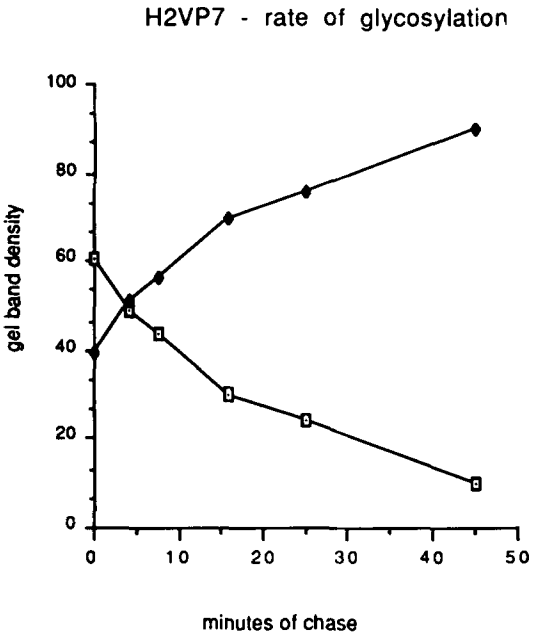

Figure 3. Kinetics of glycosylation of H2VP7. An autoradiogram similar to that shown in Fig. 2 *B* was scanned with a densitometer to quantitate the relative amounts of the two bands at the time points indicated. (□) unglycosylated VP7; (♦) glycosylated VP7.

(Fig. 4 *B*, lane *3* and *4*). These data therefore support the conclusion that some VP7 remained unglycosylated because of a delay at an early stage of its translocation into the ER. However, the size of the unglycosylated polypeptide at the time the cells were harvested suggested that its translation was complete. Therefore, a substantial portion of H2VP7 was translocated into the ER posttranslationally in vivo.

Delayed Translocation Is Not Seen with a Bovine VP7 Protein

If delayed translocation of simian VP7 is necessary for retention of the protein in the ER, then VP7 proteins from other rotavirus strains might also be expected to show similar translocation properties. Therefore, the kinetics of glycosylation (and, by inference, translocation) of VP7 from a bovine rotavirus strain (NCDV) were examined. The bovine gene used (Fig. 1, *NCDV*) contained the additional upstream, weak initiation codon present in all VP7 genes so far characterized (21). However, we have previously shown for the SA11 VP7 gene that initiation begins predominantly at Met30 and that the second (H2) hydrophobic domain functions as the signal peptide, irrespective of whether the first or second initiation codon is used (23, 29, 32). The presence of one or both hydrophobic regions also has no effect on the kinetics of SA11 VP7 glycosylation (data not shown). The NCDV gene was transfected into COS cells that were pulse-labeled for 10 min with [^{35}S]methionine and harvested after 0 or 3 h of chase. At zero chase, two bovine VP7 proteins were detected with the larger product being present in much smaller amount (Fig. 5 *A*, lane *2*; Fig. 5 *B*, lane *1*). These products were sensitive to tunicamycin (Fig. 5 *A*, lane *3*) and endo H (Fig. 5 *B*, lane *2*), indicating that both proteins were glycosylated. No unglycosylated bovine VP7 was detected after pulse-labeling (Fig. 5 *A*, compare lanes *2* and *3*), although unglycosylated simian protein was present (Fig. 5 *A*, lane *1*). Similarly, after pulse-labeling, the H2N/Sa hybrid

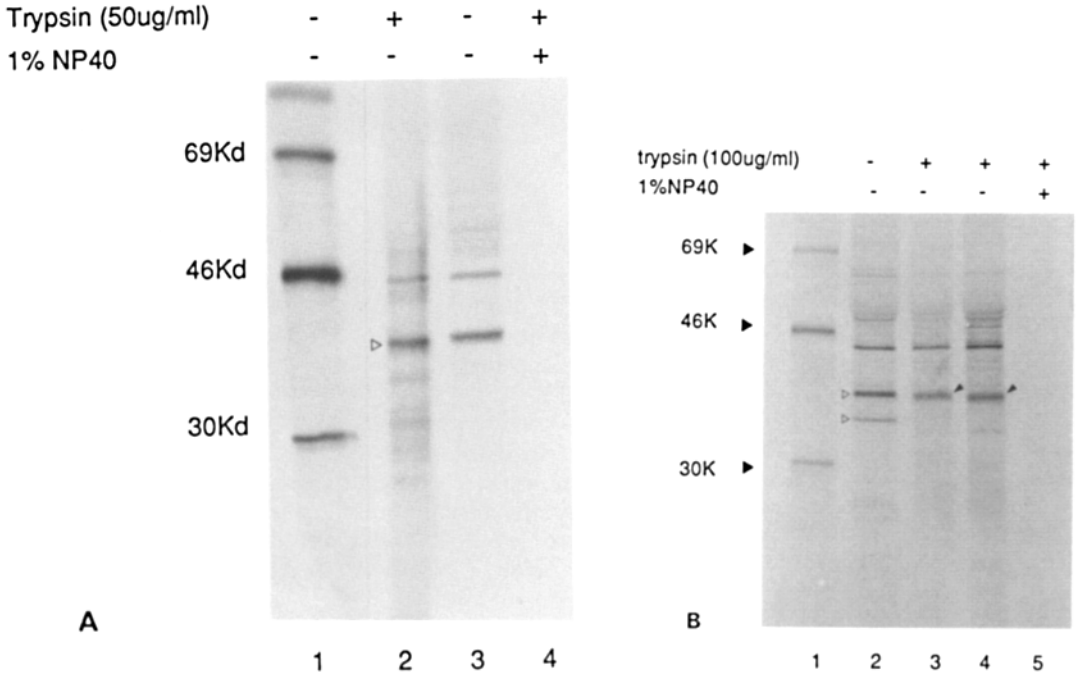

Figure 4. Analysis of proteins present in microsomes isolated from COS cells transfected with genes for (*A*) HAFVP7 and (*B*) H2VP7. Microsomes were prepared from pulse-labeled COS cells as described in Materials and Methods and incubated with (+) or without (−) NP-40 or trypsin as indicated. Microsomes in *A* were digested with trypsin on ice for 60 min. The open arrowhead indicates glycosylated HAFVP7. In *B*, open arrowheads indicate the glycosylated (*upper*) and unglycosylated (*lower*) H2VP7 proteins and solid arrowheads, the trypsin protected species after digestion for 60 min at 37°C (lane *3*) and 0°C (lane *4*). Marker proteins are run in *A* and *B*, lane *1*.

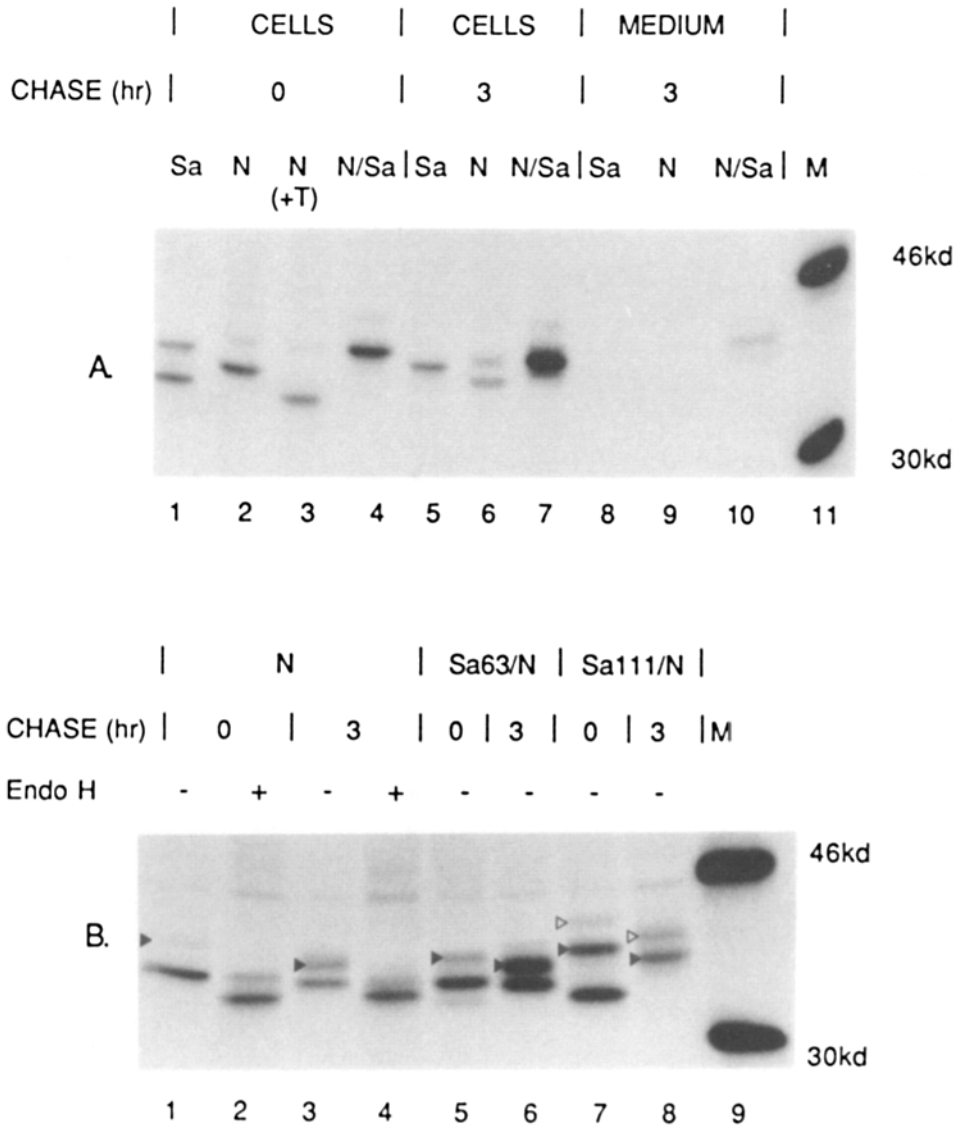

Figure 5. Glycosylation and secretion of simian, bovine, and hybrid VP7 molecules. COS cells were transfected with the appropriate genes, pulse-labeled, chased, and harvested at the times indicated. In *A*, cells were transfected with simian H2VP7 (Sa) (lanes *1* and *5*), bovine VP7 (*N*), (lanes *2* and *6*), bovine VP7 in the presence of tunicamycin (+*T*) (lane *3*), or hybrid VP7 H2N/Sa (shown as N/Sa, lanes *4* and *7*). Samples in lanes *1–4* and *5–7* were harvested at 0- and 3-h chase, respectively. Samples in lanes *8–10* were recovered from the medium of cells in lanes *5–7*, respectively. In *B*, cells were transfected with bovine VP7 (*N*) (lanes *1–4*) or with the hybrids Sa63/N (lanes *5* and *6*) or Sa111/N (lanes *7* and *8*), and harvested after a 0- or 3-h chase period, as indicated. Samples in lanes *2* and *4* were digested with endo H before analysis. Proteins glycosylated at two (▲) and three sites (△) are indicated. Marker proteins are shown in *A*, lane *11*, and in *B*, lane *9*.

protein (Fig. 1) also migrated in the position of glycosylated VP7 (Fig. 5 *A*, lane *4*). Thus, these proteins showed the normal, rapid glycosylation (and translocation) phenotype. For bovine VP7 in particular, both glycosylation sites were near the COOH terminus (Fig. 1), and, therefore, translocation must have been essentially complete. These glycosylation sites were also disproportionately used at the time labeling was terminated. However, after a 3-h chase period, the simian (Fig. 5 *A*, lane *5*), bovine (Fig. 5 *A*, lane *6*) and H2N/Sa hybrid VP7 proteins (Fig. 5 *A*, lane *7*) were all glycosylated, and the two sites on bovine VP7 (both endo H-sensitive (Fig. 5 *B*, lane *4*) were collectively ~75% filled. Despite the difference in their glycosylation phenotypes, neither simian nor bovine VP7 was secreted into the medium (Fig. 5 *A*, lanes *8* and *9*) although a small amount of the H2N/Sa hybrid was reproducibly secreted (Fig. 5 *A*, lane *10*). Delayed translocation is therefore not obligatory for the retention of all VP7 proteins in the ER.

Delayed Translocation Is Mediated by the Simian H2 Signal Peptide Plus Downstream Sequences

The difference in phenotype between the closely related bovine and simian VP7 proteins provided an opportunity to identify the simian sequences responsible for the delayed translocation of that protein. Clearly, exchange of residues 30–63 (H2N/Sa; Fig. 1), which included the H2 signal pep-

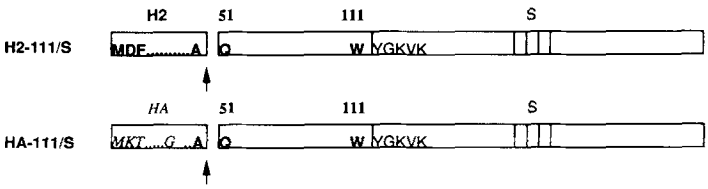

Figure 6. Structure of VP7-malaria S antigen fusion proteins with different signal peptides. VP7, HA, and S antigen sequences are in bold, italics, and plain type, respectively. Arrows indicate the signal peptide cleavage sites (28). The vertical bars indicate the repeating structure of the S antigen (10).

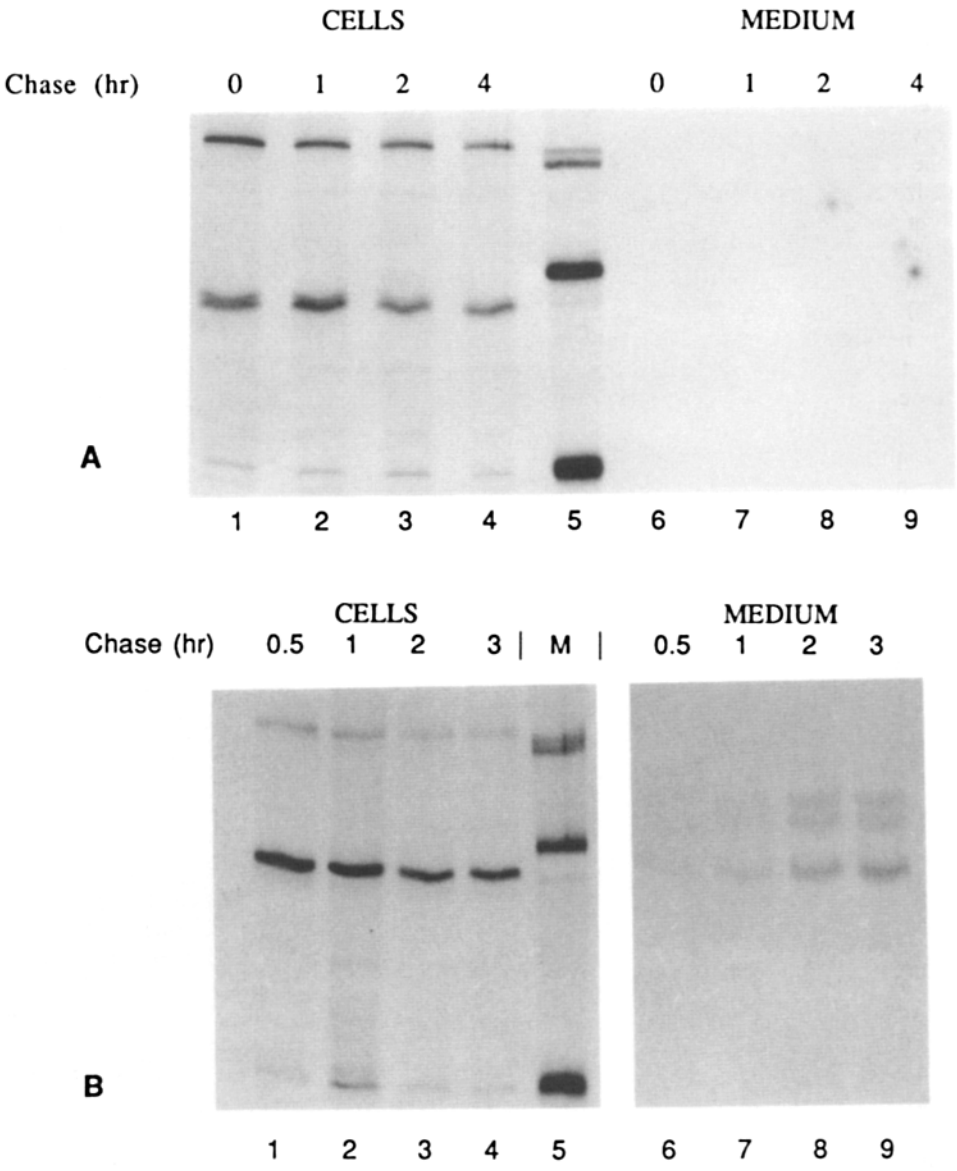

Figure 7. Synthesis and secretion of VP7-S fusion proteins by transfected COS cells. COS cells transfected with (*A*) H2-111/S were pulse-labeled with [^{35}S]methionine and chased for the times indicated. Cells and medium were harvested, and the proteins were recovered by immunoprecipitation using antiserum against S antigen. These were analyzed by gel electrophoresis and autoradiography. Samples in *B*, lanes *6–9* were run on a separate gel with the same marker proteins.

tide regions and a conservative Ile-Val change at residue 55, was sufficient to convert simian VP7 from a slow to a fast translocation phenotype (Fig. 5 *A*, lane *4*). In the reciprocal experiment in which the bovine protein was the reporter molecule, attachment of the simian H2 region (Sa63/N; Fig. 1) did not alter the rate of glycosylation appreciably. As for the intact bovine VP7 (Fig. 5 *B*, lane *1*), the two available sites in the hybrid protein were rapidly, but, disproportionately, filled at the end of the labeling period and little unglycosylated product was detectable (Fig. 5 *B*, lane *5*). Again, following the 3-h chase period, the two glycosylation sites were ~75% filled (Fig. 5 *B*, lanes *3* and *6*). The presence of additional simian residues (64–111), including the simian glycosylation site at Asn 69 (Sa111/N, Fig. 1), was sufficient to convert the bovine protein from the fast to the slow translocation phenotype. At least 50% of the hybrid protein remained unglycosylated after pulse-labeling (Fig. 5 *B*, compare lanes *1* and *5* with *7*), and this unglycosylated protein could be recovered in microsomes and was trypsin sensitive (data not shown). Again, judging from the polypeptide mobilities, at the end of the labeling period, two of the three available glycosylation sites were preferentially used (Fig. 5 *B*, lane *7*), but, by the end of the chase period, the third, presumably COOH-terminal site was also partially filled (Fig. 5 *B*, lane *8*). Judging from the migration of these proteins at 0 and 3 h, there was also evidence of carbohydrate trimming that is known to occur (18) (Fig. 5 *B*, lanes *1*, *3*, *5*, *6*, *7*, and *8*). Thus, two regions of the simian VP7 protein, the H2 signal peptide and amino acids residues 64–111 inclusive, appear to be involved in conferring the slow glycosylation/translocation properties on the nascent polypeptide.

Retention of Simian VP7 Is Also Mediated by the Signal Peptide Plus Residues 64–111

It was previously shown that the H2 signal peptide plus sequences in the mature portion were required to retain VP7 in the ER (28). The downstream sequences were identified by splicing a larger portion of VP7 onto the normally secreted malaria S antigen (10) (Fig. 6, *H2-111/S*). COS cells transfected with this hybrid gene were pulse-labeled for 30

min with [^{35}S]methionine and then chased for up to 4 h. Expressed proteins, recovered by immunoprecipitation with S antiserum from the cells and the medium, were analyzed by polyacrylamide gel electrophoresis (Fig. 7 *A*). After the labeling period, two polypeptides were visible in the cells (Fig. 7 *A*, lane *1*), but the upper one disappeared after a 2-h chase (Fig. 7 *A*, lanes *1–3*). The size of the lower product was also reduced in the presence of tunicamycin (data not shown), indicating that it was glycosylated. This was expected since the VP7 region attached contained an N-linked glycosylation site at Asn69 (Fig. 6). However, the hybrid protein was not detected in the medium even after a 4-h chase period (Fig. 7 *A*, lanes *6–9*), suggesting that the sequences necessary for retention in the ER were present in the VP7 portion.

To confirm that this retention was specific and that the hybrid protein behaved in the same manner as intact VP7, the HA signal peptide was substituted for the signal sequence in H2-111/S such that the same cleavage site was conserved (28) (Fig. 6, *HA-111/S*). When this hybrid gene was expressed in a pulse-chase experiment, the protein was clearly detectable in the cells and the medium (Fig. 7 *B*, lanes *1–4* and *6–9*, respectively). Some of the secreted protein had a higher molecular weight, consistent with the addition of complex carbohydrate to the molecule during transport. Thus, the H2 signal peptide and the first 60 residues of mature VP7 together are necessary and sufficient for retention of the hybrid S antigen and VP7 in the ER.

Discussion

The data presented here confirm and extend our initial observation with COS cells that glycosylation of the simian rotavirus VP7 protein, a resident of the ER, occurs more slowly than glycosylation of its secreted counterpart, which is produced from a precursor with a different signal peptide (28). Here, we showed that the slow rate of glycosylation is caused by a rate limiting step at an early stage during translocation of this protein into the ER, such that the rate of translation exceeds that of translocation. The majority of newly synthesized H2VP7 (35.5-kD total size) is therefore taken up posttranslationally in vivo. To our knowledge, posttranslational translocation has only been observed previously in vitro for proteins that are still attached to ribosomes, for small proteins whose uptake is signal recognition particle independent, and for some yeast proteins that are targeted to the ER (for review, see references 26 and 30). In this work we did not attempt to determine whether ribosomes were still attached to untranslocated VP7 in vivo. However, it seems likely that polypeptide chain termination and ribosome release would occur in vivo unless a feedback control between the translocation and protein synthetic machinery exists to prevent this. By splicing simian sequences onto a bovine VP7 protein, we showed that the H2 signal peptide of simian VP7 together with amino acids 64–111 of the mature protein were both required to produce delayed translocation. Since a VP7 mutant in which residues 51–62 were deleted (23) also shows this phenotype (our unpublished results), the necessary sequences must be wholly contained within these two regions. Attenuation of translocation was previously observed in vivo for hybrid proteins carrying mutations at the NH_2 terminus (15). Transposition of amino terminal tripeptides in the signal peptide of pre-pro-alpha factor–somatostatin hybrids caused a 45–75% reduction in the efficiency of translocation of these proteins, which was arrested before signal peptide cleavage and glycosylation. In another study also involving the pre-pro-alpha factor signal sequence, replacement of a residue in the hydrophobic core with a proline or hydrophilic residue decreased the rate of translocation in vitro up to 50-fold (1). The situation here is different for several reasons. First, delayed, posttranslational translocation of simian VP7 appears to be the normal situation for this protein in COS cells. Second, translocation is delayed after signal peptide cleavage since the uncleaved precursor of H2VP7 cannot be detected in the cell (28). Third, the bovine and simian VP7 proteins have almost identical sequences at the NH_2 terminus of their respective H2 signal peptides (Fig. 1), suggesting that this region alone is not responsible for the effect. Finally, two regions of the simian protein are required to produce the phenotype. If the H2 signal peptide and (part of) residues 64–111 formed a loop structure for insertion into the membrane prior to cleavage (11, 27), these two regions might then interact within the same proteinaceous channel (6) or membrane pore (33) to modulate translocation of the protein. This is consistent with our earlier proposal that suggested that the H2 signal peptide may normally be sequestered by an interaction with another (hydrophobic) region of VP7, thereby preventing inappropriate interactions during translocation (28). Presumably, there is a higher energy barrier to translocation for the simian protein, compared with bovine VP7, which accounts for the delay (11). A difference in the extent of glycosylation was also observed after a 0- or 3-h chase period (Fig. 5 *B*) for bovine proteins that have a naturally occurring glycosylation site at Asn318; i.e., 8 residues from the COOH terminus of VP7 (Fig. 1). This seems likely to be due to an underutilization of this site, perhaps because it is too close to the COOH terminus to be used efficiently. Alternatively, it is not known how VP7 is anchored although it is classified as a membrane-associated protein (18, 19). There is a generally hydrophobic sequence in VP7 (amino acids 292–310) that also contains two Asp residues (17). If this region were a "pseudo" transmembrane anchor domain that temporarily anchored the protein, the COOH terminal glycosylation site might have taken some time to be translocated to the lumenal side where glycosylation occurs. In that case, it may be possible to detect a difference in the integral membrane status of the protein after a long chase period. Slow release of VP7 from the membrane might also assist its incorporation into the outer capsid layer of the nonenveloped rotavirus particle.

In an earlier study, we showed that the H2 signal peptide together with downstream sequences was required for retention of SA11 VP7 in the ER (28). Here we have shown further that sequences within the first 60 residues of mature VP7, together with the signal peptide, are necessary and sufficient for retention of the protein in the ER. These conclusions are partly in agreement with an earlier study that showed using enzymatically active VP7/α-amylase chimeras that residues 62–111 were required for retention (22). However, this study also concluded that residues 51–61 formed part of the retention signal. This is inconsistent with two observations that: (*a*) the VP7 deletion mutant 51–61 was not secreted (22); and (*b*) VP7 derived from the HAFVP7 precursor was secreted, although it contains these residues (28). Thus, we conclude

that the H2 signal peptide plus residues 64–111 together comprise the retention signal. Also, consistent with this is the observation that the hybrid molecule H2N/Sa was retained in the cells to a lesser degree than the wild-type simian or bovine proteins (Fig. 5 *A*, lanes *8–10*), perhaps because an interaction between the H2 signal peptide and residues 64–111 was partially disrupted by the exchange of the bovine and simian signals whose sequences vary.

Thus, the sequences that are required for retention also mediate an effect on the translocation of simian VP7. However, the observation that the bovine VP7 protein was rapidly glycosylated and translocated, and yet still retained in the ER makes it clear that the delay in translocation seen for the simian protein was not a general requirement for retention of other VP7 molecules in the ER. Rather, it seems as though the simian protein simply translocates in "slow motion" relative to the bovine protein. This may provide an opportunity for a more detailed examination of the process of translocation and the cellular components involved.

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