Differential Effects of Changes in the Length of a Signal/Anchor Domain on Membrane Insertion, Subunit Assembly, and Intracellular Transport of a Type II Integral Membrane Protein*

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The length requirement for a functional uncleaved signal/anchor (S/A) domain of the paramyxovirus hemagglutinin-neuraminidase (HN) type II glycoprotein was analyzed. HN mutants with progressive NH₂-terminal S/A deletions or insertions were expressed in HeLa cells, and the membrane targeting, folding, tetramer assembly, and intracellular transport of the proteins were examined. Changing the length of the S/A by two residues resulted in HN mutants that displayed aberrant endoplasmic reticulum (ER) membrane targeting or translocation. This phenotype did not simply reflect upper or lower limitations on the size of a functional S/A, because normal signaling was restored by further alterations involving three or four residues. Likewise, ER-to-Golgi transport of mutants containing deletions of one or two S/A residues was delayed (~30% of WT) or blocked, but transport was restored for a mutant with a total of three deleted residues. HN mutants with S/A insertions of three or four Leu residues differed from wild-type HN by having heterogeneous Golgi-specific carbohydrate modifications. Differences in ER-to-Golgi transport of the mutants did not strictly correlate with defects in either native folding of the ectodomain or the assembly of two dimers into a tetramer. Together, these data suggest that efficient entry into and exit from the ER are sensitive to changes in the HN S/A that may reflect alterations to a structural requirement along one side of an α-helix.

The synthesis and assembly of eukaryotic integral membrane proteins differs in several ways from the biosynthesis of other types of cellular proteins. A specific requirement for the biosynthesis of a membrane protein is that the nascent polypeptide chain must first be targeted to the endoplasmic reticulum (ER)³ through interactions with a dedicated cellular machinery (reviewed in Walter and Lingappa (1986)). Once the nascent chain has been inserted into the lipid bilayer, additional unique aspects of the biosynthesis of membrane proteins involve the folding and assembly of distinct extracellular, cytoplasmic, and lipid-imbedded regions of the protein. Thus, individual segments of a single polypeptide chain must undergo maturation steps in very different environments, including the ER lumen, the cytosol, and the hydrophobic lipid bilayer.

Proper folding of these individual domains and subunit assembly are important factors that influence the intracellular transport of membrane proteins. An ER-localized mechanism appears to act on newly synthesized integral membrane proteins to control ER-to-Golgi transport (reviewed in Doms et al. (1993), Hammond and Helenius (1995), and Pelham (1989)). For a large number of membrane proteins, progress has been made in our understanding of some of the factors and biosynthetic steps involving the ER-to-lumen ectodomain that dictate the formation of a transport-competent protein, and ER proteins that assist in these steps have been identified (Hammond and Helenius, 1995; Pelham, 1989).

Likewise, a number of studies on the cytoplasmic tail of membrane proteins have identified a variety of distinct signals in this domain that act during biosynthesis and intracellular transport (e.g. Casanova et al. (1991), Nilsson et al. (1989), and Parks and Lamb (1993)). In contrast to the ecto- and cytoplasmic domains, the questions of how lipid-imbedded segments influence membrane protein biosynthesis and the nature of structural requirements in this domain remain largely unanswered.

For the majority of known integral membrane proteins, an NH₂-terminal cleavable sequence serves to signal the nascent chain to the ER membrane and a separate COOH-terminal transmembrane (TM) domain acts as a membrane anchor. By contrast, the type II integral membrane proteins lack an NH₂-terminal cleavable signal sequence, but contain an internal hydrophobic signal/anchor (S/A) domain that serves a dual function: the signaling of the nascent polypeptide to the ER membrane and the subsequent anchoring of the polypeptide in the lipid bilayer. Examples of type II proteins include the asialoglycoprotein receptor (Spiess and Lodish, 1986), the influenza virus neuraminidase (Boo et al., 1984), signal peptidase (Sheldes et al., 1993), the Golgi-resident glycosyltransferases (Paulson and Colley, 1989), and the paramyxovirus hemagglutinin-neuraminidase (HN) glycoprotein (Hiebert et al., 1985).

The HN protein of the paramyxovirus simian virus 5 has served as a model type II integral membrane protein for the analysis of the membrane insertion, assembly, and intracellular transport (Ng et al., 1989, 1990; Parks and Lamb, 1990a, 1990b, 1993). The predicted structure of the simian virus 5 HN protein includes a 17-residue cytoplasmic tail, a 19-amino acid uncleaved S/A, and a large 523 residue COOH-terminal ectodomain (Hiebert et al., 1985). Newly-synthesized HN folds into a structure that is recognized by conformation-specific antibodies and oligomerizes into a tetramer (t₁⁄₂ ~ 25–30 min) before trans-
port from the ER to the medial Golgi (Hiebert et al. previously (Parks, 1994; Parks and Pohlmann, 1995) using a 5' template for oligonucleotide-directed mutagenesis exactly as described.

Recent work has shown that the uncleaved S/A domain serves multiple functions in the biosynthesis of a type II protein, including ER signaling, membrane anchoring, subunit assembly, and intracellular targeting. A typical S/A domain is longer than the hydrophobic core of a cleavable signal sequence (von Heijne, 1988), suggesting that subdomains may exist within the S/A that direct these individual functions. Previous attempts to identify by deletion analysis the subdomains that are responsible for signaling and anchoring have led to the proposal that these two functions are encoded by redundant and overlapping segments within the S/A (Brown et al., 1988; Lipp and Dobberstein, 1988; Spiess and Handschin, 1987; reviewed in Nayak and J abbar, 1989). In contrast, mutational analysis of the HN protein has identified residues at the COOH-terminal end of the S/A that are important for the assembly of a tetramer from two dimers (McGinnnes et al., 1993; Parks and Pohlmann, 1995), suggesting that the tetramer assembly function may reside in a subdomain of the HN S/A.

It has been shown that S/A alterations can also influence the intracellular transport of a type II protein (reviewed in Machamer, 1993; Nayak and J abbar, 1989), but to date no unifying theory has been developed for the role that the S/A domain serves in the formation of a protein that is competent for transport from the ER. Although it is proposed that ~20 amino acids are sufficient to span a lipid bilayer as an α-helix, comparisons of known type II proteins have shown a wide range of S/A lengths (Hartmann et al., 1989; Bretscher and Munro, 1991). In some cases, the length of a S/A and not the specific amino acid sequence per se may be an important aspect of intracellular targeting of type II proteins (Masibay et al., 1993; Munro, 1991). In this report, a systematic approach has been taken to analyze the influence of S/A length on the biosynthesis and intracellular transport of HN, a model type II plasma membrane protein. The results suggest that efficient entry into and exit from the ER are sensitive to changes in the conformation of the HN S/A that may reflect alterations to a structural requirement along one side of an α-helix.

**MATERIALS AND METHODS**

Plasmid Construction and Mutagenesis—An EcoRI-PstI fragment of the simian virus 5 HN protein gene (encoding HN residues 1–81, Hiebert et al. (1985)) in pGem3 (Promega, Madison, WI) was used as template for oligonucleotide-directed mutagenesis exactly as described previously (Parks, 1994; Parks and Pohlmann, 1995) using a 5' mRNA-sense mutagenic oligonucleotide along with primers specific for the T7 and SP6 promoter sequence contained in pGem3. The resulting 280-base pair polymerase chain reaction product was digested with EcoRI and PstI before ligation into the corresponding sites of pGem3 such that mRNA-sense transcripts could be produced using the T7 RNA polymerase promoter. The nucleotide sequence of the entire polymerase chain reaction-derived DNA fragment was determined by dideoxy nucleotide sequencing. To reconstruct the full-length HN gene, plasmid DNA encoding the desired mutation was digested with PstI, phosphatase treated, and ligated with a DNA fragment encoding the WT HN ectodomain (residues 82–565).

Cells—Monolayer cultures of HeLa T4 cells (Madden et al., 1986) were grown in Dulbecco's modified Eagle's medium containing 10% fetal calf serum.

Isotopic Labeling of Polypeptides, Immunoprecipitation, Endoglycosidase Treatment, and Polyacrylamide Gel Electrophoresis—Proteins were expressed in HeLa T4 cells from cDNA plasmids as described (Parks, 1994) using a modified version of the vaccinia virus/T7 RNA polymerase system of Fuerst et al. (1996). Briefly, 24-well dishes of subconfluent cells were infected with vTF7.3, a recombinant vaccinia virus that expresses T7 RNA polymerase (multiplicity of infection ~10). Infected cells were then transfected with 0.5 μg of pGEM plasmid DNA encoding the HN mutants along with 5 μg of Lipofectin reagent in 200 μl of Opti-MEM (Life Technologies, Inc., Gaithersburg, MD). All media contained 100 μg/ml cytosine β-arabinofuranoside (AraC, Sigma) to inhibit the growth of vaccinia virus. Cells were radiolabeled at 4.0 h post-transfection with 100–200 μCi/ml Tran35S-label (ICN Radiochemicals Inc., Irvine, CA) in Dulbecco's modified Eagle's medium lacking cytosine and methionine as described previously (Parks and Pohlmann, 1995). In chase protocols, radiolabeled cells were washed twice with Dulbecco's modified Eagle's medium supplemented with 2 μM nonradioactive methionine (chase medium), and then incubated in the medium for the indicated time.

Cells were washed in phosphate-buffered saline, lysed in 1% SDS, boiled for 5 min, and clarified by centrifugation (5,000 × g). Immunoprecipitations were carried out as described previously (Ng et al., 1989; Lamb et al., 1978), using rabbit polyclonal antiserum raised to SDS-denatured HN (HN antisera, Ng et al. (1990)). Alternatively, cell lysates were immunoprecipitated with mouse monoclonal antibodies (mAbs) 1b and 4b that recognize epitopes in the fully mature form of HN (Randall et al., 1987). Following immunoprecipitation, samples were treated with endo-β-N-acetylglucosaminidase H (endonH) or peptidase-N-glycosidase F (N-glycanase) as described by the manufacturer (New England Biolabs, Beverly, MA). Samples were analyzed by SDS-PAGE on 10% polyacrylamide gels followed by autoradiography (Lamb and Choppin, 1976). Radioactivity in dried gels was quantitated using an Ambis radiodensitometric imaging system (San Diego, CA). Acquisition of the endoH-resistant carbohydrate-resistant form of HN was expressed as the ratio of radioactivity in the endoH-resistant form divided by the total HN protein in both the endoH-resistant and -sensitive forms. The data are the average of at least two experiments (±10%), and are expressed as a percentage of that obtained with WT HN assayed in parallel.

Sucrose Density Gradient Sedimentation Analysis—Vaccinia virus vTF7.3-infected HeLa T4 cells (35 mm dishes) were transfected with plasmids (5 μg) encoding mutant HN proteins and radiolabeled for 15 min with 100 μCi/ml Tran35S-label as described above. Cells were incubated in chase medium for 90 min. The oligomeric form of the HN mutants was analyzed by sucrose gradient sedimentation as described previously (Ng et al., 1989; Parks and Pohlmann, 1995). Protein standards (rabbit hemoglobin, ~45, and potato β-amylase, ~95) were analyzed in parallel gradients and fractions were analyzed by SDS-PAGE followed by Coomassie Blue staining.

Indirect Immunofluorescence—3.5-cm dishes of vTF7.3-infected HeLa T4 cells were transfected with plasmids encoding the HN mutants as described above and processed at 15 h post-transfection for indirect cell surface immunofluorescence using mAb 4b (Randall et al., 1987) as described previously (Parks and Lamb, 1990a).

**RESULTS**

Expression of HN NH2-terminal S/A Mutants—To determine how S/A length influences the biosynthesis of HN, the NH2-terminal end of the HN S/A was modified by progressive single amino acid deletions to create mutants Del N1–4 (Deletion, N-terminal, 1–4 residues, Fig. 1). Mutant proteins were expressed to high levels by first infecting HeLa T4 cells with recombinant vaccinia virus that synthesizes bacteriophage T7 RNA polymerase (Fuerst et al., 1986) and then transfecting the cells with DNA plasmids encoding the HN mutants under control of the T7 promoter. Infected/transfected cells were then radiolabeled with Tran35S-label for 30 min and proteins were immunoprecipitated from cell extracts with antisera that recognizes all forms of HN before analysis by SDS-PAGE. WT HN was synthesized as a single major polypeptide of ~68 kDa (Fig. 2, WT lane) and was converted to a faster migrating form after deglycosylation by treatment with N-glycanase (HN0, D lane). HN proteins containing deletions of one or two residues from the NH2-terminal end of the S/A (Del N1 and Del N2) were detected as polypeptides with an electrophoretic mobility matching that of WT HN (Fig. 2, deletion lanes 1 and 2). In the case of Del N2, however, approximately 20% of the newly-synthesized protein was detected as an additional form that had a mobility on gels matching that of deglycosylated HN0 (deletion panel, lane 2). Digestion of Del N2 protein with N-glycanase resulted in a single HN0 species on SDS gels (data...
not shown, but see below), indicating that the HN and HNo forms of Del N2 were a single polypeptide chain that migrated differently on SDS gels due to the presence and absence of carbohydrate chains, respectively. As the only sites for N-linked glycosylation of HN are in the COOH-terminal ectodomain, the presence of the unglycosylated HN0 form of mutant Del N2 indicates that this protein is partially defective in some aspect of ER targeting or translocation across the ER membrane. To determine if aberrant glycosylation seen with mutant Del N2 was not simply a reflection of a lower limit on the size of a functional HN S/A, deletion panel, lanes 3 and 4). These data indicate that the aberrant glycosylation seen with mutant Del N2 was not simply a reflection of a lower limit on the size of a functional HN S/A.

To determine if S/A insertions had a similar effect on HN biosynthesis, HN mutants Ins N1-4 (Ins N1–4 residues, Fig. 1) were constructed in which one to four Leu residues were inserted into the NH2-terminal end of the S/A. When expressed using the vaccinia virus/T7 system described above, all of the insertion mutant produced a protein that comigrated on SDS gels with WT HN (Fig. 2, Insertion panel, lanes 1–4). Most importantly, the insertion of two additional Leu residues into the S/A resulted in a mutant (Ins N2) in which nearly half of the newly-synthesized protein was detected as the unglycosylated HN0 form (Insertion panel, lane 2). In contrast, mutations with insertions of one, three, or four Leu residues produced relatively little of the HN0 form (~5% of total HN protein). The finding that insertions or deletions of two S/A residues results in an unglycosylated form of HN suggests a phase-specific requirement in the HN S/A for efficient ER targeting or translocation (see below). Treatment of membranes isolated from cells expressing the Ins N2 mutant protein with pH 11.0 buffer did not extract the HN0 polypeptide into a soluble form (data not shown), suggesting that this protein species was stably associated with membranes (Steck and Yu, 1973). It is possible that the deletion or insertion of two residues into the S/A leads to molecules that are partially defective in ER targeting or translocation, such that the COOH-terminal domain of these molecules, which contains the only sites for glycosylation, had not been translocated into the ER lumen.

Previous work has demonstrated that alterations to charged residues flanking the HN S/A can lead to molecules that are inserted into the ER membrane but have an inverted transmembrane topology (Parks and Lamb, 1993). Thus, a possible explanation for the presence of the faster migrating HN0 form of the Del N2 and Ins N2 mutants is that they represent molecules whose transmembrane orientation has been reversed, such that they are now anchored in the membrane with the NH2-terminal domain in the ER lumen. A prediction of this outcome is that translocation of the NH2-terminal domain of the inverted molecules across the ER membrane should expose the NH2 terminus to the glycosylation machinery that resides in the ER lumen. To test this possibility, a consensus site for N-linked glycosylation (Asn-Ala-Thr; Parks and Lamb (1993)) was added by site-specific mutagenesis to the NH2-terminal domain of mutant Ins N2 to create Ins N2* (see asterisk in Fig. 1 for location). A previously characterized HN mutant containing substitutions of glutamate for arginine 13 and arginine 17 on the cytoplasmic side of the S/A (see Fig. 1) was expressed as a positive control for inversion of HN membrane topology, as this mutant adopts two opposing transmembrane topologies due to these amino acid changes (HN 18*, Parks and Lamb (1993)). Following expression in HeLa cells and radiolabeling with Tran35S-label, HN proteins were immunoprecipitated, treated with endoglycosidase, and analyzed by SDS-PAGE. The addition of carbohydrate to the NH2-terminal glycosylation site of the inverted HN 18* molecules (Fig. 3, lane 2, HN18*) results in a slower electrophoretic mobility than deglycosylated HN (+ lane 2). The mobility of the HN species is not altered by the presence of the new glycosylation site, because the NH2 terminus is in the cytoplasm.

Ins N2* was detected in equal amounts as two major polypeptide species (Fig. 3, lane 1): one form with an electrophoretic mobility matching fully glycosylated WT HN and a second form with a mobility matching the deglycosylated pro-
tein (HN_o). The comigration of the faster migrating Ins N2* species with deglycosylated HN indicates that the new NH2-terminal glycosylation site in the Ins N2* mutant was not utilized. Given the caveat that alterations to the S/A may have prevented glycosylation of a fully-translocated NH2-terminal domain of Ins N2* molecules, these data indicate that the HN_o form seen with mutants Del N2 and Ins N2 is not due to bona fide inversion of the normal HN transmembrane orientation. While the nature of the unglycosylated form of these mutants was not explored further, the insertion or deletion of two residues at the NH2-terminal end of the S/A results in polypeptides that are aberrantly targeted to or translocated across the ER membrane.

Intracellular Transport of HN S/A Mutants—The transport of the HN S/A mutants from the ER was examined to determine if changes in the length of the HN S/A affected biosynthetic steps subsequent to ER membrane targeting and insertion. The HN mutants were expressed in HeLa cells as described above and radiolabeled for 30 min with Tran35S-label. Cells were then incubated in chase medium for 90 min, the half-time for transport of HN to the medial Golgi (Ng et al., 1989). To monitor ER-to-Golgi transport of the mutants, the conversion of COOH-terminal glycans from a high-mannose ER form to a complex Golgi-specific form was assayed by treating immune complexes with endoH before SDS-PAGE. Immune complexes were then divided into two aliquots which were incubated in nonradioactive chase medium for 90 min. Proteins were immunoprecipitated from cell lysates with HN antiserum. Immune complexes were incubated with (+lanes) or without (−lanes) N-glycanase before analysis by SDS-PAGE. The positions of WT (HN), deglycosylated (HN_o), and inverted (HN_NH2-endo) forms of HN are indicated by arrows.

![Image](https://example.com/image1.png)

**Fig. 3.** The NH2 terminus of HN mutant Ins N2 is not translocated into the ER lumen. vTF7.3 infected HeLa T4 cells were transfected with plasmid DNA encoding a modified version of mutant Ins N2 (Ins N2*, lane 1) or with a charge-altered HN mutant that adopts two opposing transmembrane orientations (mutant HN18*, lane 2). Both proteins contained a site for the addition of N-linked carbohydrate at the NH2 terminus. Following a 30-min radiolabeling with Tran35S-label, cells were lysed and proteins immunoprecipitated with anti-HN serum. Immune complexes were incubated with (+lanes) or without (−lanes) N-glycanase before analysis by SDS-PAGE. The positions of WT (HN), deglycosylated (HN_o), and inverted (HN_NH2-endo) forms of HN are indicated by arrows.

![Image](https://example.com/image2.png)

**Fig. 4.** Acquisition of endoH resistance for HN S/A mutants. HeLa T4 cells were infected with vTF7.3 and then transfected with plasmid DNA encoding wild type HN (WT lanes), one of the NH2-terminal deletion mutants Del N1-4 (panel A, lanes 1-4, respectively), or one of the HN Leu insertion mutants Ins N1-4 (panel B, lanes 1-4, respectively). Cells were radiolabeled with Tran35S-label, and incubated in nonradioactive chase medium for 90 min. Proteins were immunoprecipitated from cell lysates with HN antiserum. Immune complexes were then divided into two aliquots which were incubated with buffer alone (−lanes) or with endoH (+lanes) before analysis of samples by SDS-PAGE. R, endoH-resistant HN species; S, endoH-sensitive HN species. The asterisk in panel B indicates the position of a variant form of endoH-resistant HN.

Glycans by 90 min after synthesis (Fig. 4A, compare WT, 1 and 2 + lanes). Quantitation of gels from multiple experiments indicated that the fraction of the total Del N1 protein that had accumulated endoH-resistant carbohydrates (i.e. the ratio of the resistant form to total radiolabeled protein) was only ∼30% of the value obtained for WT HN. In the case of Del N2, only trace amounts of protein with endoH-resistant glycans were detected. These data indicate that decreasing the HN S/A by one or two residues can reduce or block ER-to-Golgi transport.

Surprisingly, however, a further deletion that removed a total of three consecutive residues from the NH2-terminal end of the S/A resulted in a mutant protein (Del’N3) that was competent for transport from the ER (Fig. 4A, + lane 3). Quantitation of gels from multiple experiments indicated that the fraction of the total Del N3 protein that had accumulated endoH-resistant carbohydrates was on average ∼70% of the value found with WT HN analyzed in parallel. The removal of a total of four S/A residues resulted in a mutant that accumulated only trace amounts of endoH-resistant glycans (+ lane 4). Thus, transport of HN from the ER is reduced or blocked by removal of one, two, or four consecutive residues from the NH2-terminal end of the S/A, but transport can be restored to...
levels similar to that obtained with WT HN when a total of three residues are deleted from this region.

The effect of extending the length of the HN S/A on ER-to-Golgi transport was examined as described for the deletion mutants above. HN mutants containing insertions of one or three Leu residues into the NH2-terminal end of the S/A accumulated endoH-resistant glycans to approximately half the level seen with WT HN (Fig. 4A, + lanes 1 and 3). In the case of mutants with insertions of two or four consecutive Leu residues, the fraction of properly translocated HN molecules was significantly lower than that seen for mutants with insertions of one or three Leu residues (+ lanes 2 and 4). As transport of these insertion mutants was not blocked, these data suggest that the overall effect of insertions into the S/A was less disruptive to ER-to-Golgi transport than were the deletions described above.

Close inspection of the products resulting from endoH digestion of mutants Ins N3 and Ins N4 showed an additional protein species that migrated between the R and S forms of HN (asterisk, Fig. 4B). By comparison to the products of a partial digestion of WT HN produced by using limiting amounts of endoH, the mobility of this endoH-resistant variant form was consistent with HN polypeptides in which only one of the four carbohydrate chains had been processed to an endoH-resistant form (not shown). A time course of acquisition of endoH resistance was carried out for Ins N3 and Ins N4 to determine the kinetics of carbohydrate processing. As shown in Fig. 5, the variant form of endoH-resistant HN was first detected by 30 min after synthesis for both Ins N3 and Ins N4, and this species accumulated with approximately the same kinetics as the fully processed R form. In comparison, WT HN was processed throughout the time course into two distinct forms (R and S arrows, Fig. 5, WT panel), with no detectable levels of the variant endoH-resistant form. Thus, in contrast to the S/A deletions described above, insertions into the NH2-terminal end of the S/A do not block ER-to-Golgi transport. However, insertions of three or four residues appear to alter the processing of HN COOH-terminal carbohydrates by Golgi enzymes.

Oligomeric Form of HN S/A Mutants—HN is a homotetramer composed of a pair of disulfide-linked dimers that associate by noncovalent bonds (Ng et al., 1989). The different ER-to-Golgi transport characteristics shown by the S/A deletion mutants could reflect differences in assembly of HN into a homotetramer, a step that occurs prior to transport of HN to the Golgi (Ng et al., 1989). To determine the oligomeric form of the S/A mutants, HeLa cells expressing the deletion mutants were pulse-labeled for 15 min with Tran35S-label and incubated for 90 min with nonradioactive chase media. Cell lysates were prepared and analyzed by sucrose gradient sedimentation as described under "Materials and Methods." Gradient fractions were analyzed by immunoprecipitation and SDS-PAGE under nonreducing conditions. The P lane represents proteins immunoprecipitated from the pellet fraction that was recovered from the bottom of the tube. Sedimentation was from right (top fraction) to left (bottom fraction). HN1, monomer; HN2, disulfide-linked dimer. Arrows in panel A denote the positions of 9 S and 4 S sedimentation markers that were analyzed in a parallel gradient.

As shown in Fig. 6, WT HN was detected on sucrose gradients as a major disulfide-linked species sedimenting slightly slower than a 9 S marker protein (panel A, fractions 5 and 6). The HN protein in the 9 S region of the gradient migrates on SDS gels as a disulfide-linked dimer (HN2), consistent with the proposal that two dimers are held in a tetramer by noncovalent interactions. A small amount of disulfide-linked HN was detected near the bottom of the gradient (fraction 1) and also as an aggregate in the pellet fraction that was too large to effectively enter the gel (P fraction). HN mutants Del N1-N4 were all detected on sucrose gradients as slower sedimenting disul-
fide-linked dimers (panels B-E, respectively, fractions 8 and 9), indicating that each of these mutants was defective in the assembly of two dimers into a stable tetramer. It is noteworthy that the NH₂-terminal deletion mutants also produced significant levels of monomeric HN protein (e.g. Fig. 6, panels C and E), a result that suggests an additional role for this region of the S/A in the assembly of two HN monomers into a dimer. The finding that mutants Del N2 and Del N3 both sedimented as dimers (panels C and D) and yet displayed very different ER-to-Golgi transport phenotypes indicates that the efficiency with which a S/A mutant is transported from the ER does not strictly correlate the assembly of two dimers into a stable tetramer.

The oligomeric form of the HN insertion mutants Ins N1–4 was determined by sucrose gradient sedimentation. For each insertion mutant, the major oligomeric form detected on sucrose gradients was a disulfide-linked dimer (Fig. 7, fractions 8 and 9) that sedimented more slowly than the WT HN tetramer. In addition, lesser amounts of insertion mutants Ins N2-N4 were detected as higher order disulfide-linked oligomers that sedimented to the bottom portion of the gradient (panels B, C, and D, fractions 1-5). A portion of the mutant polypeptides displayed aberrant electrophoretic mobilities under the nonreducing conditions of the SDS gels (e.g. Fig. 7, panel D, HN2 species), a result suggesting that disulfide bonding of the large COOH-terminal ectodomain of these mutants was altered. Together these results indicate that the formation of a stable HN tetramer is sensitive to deletion or insertion of even a single residue from the NH₂-terminal end of the S/A, and that tetramer assembly defects do not correlate with efficiency of transport from the ER.

Folding and Cell Surface Expression of S/A Mutants—Previous work has shown that the folding of the simian virus 5 HN to a form recognized by conformation-specific mAbs correlates with transport of newly-synthesized protein from the ER, and that alterations in proper folding of the COOH-terminal ectodomain can lead to blocks in ER-to-Golgi transport (Ng et al., 1989). The S/A mutants showed no detectable defect in folding of the COOH-terminal ectodomain as assayed by their reactivity with conformation-specific HN mAbs (summarized in Table I). The only exception to this was found with mutants Del N2 and Ins N2. For these mutants, a large fraction of the newly-synthesized protein was produced as the unglycosylated HNo (see Fig. 2, above), a form of HN that is not recognized by the HN conformation-specific mAb (Ng et al., 1989). However, the fraction of HN containing endoH-resistant carbohydrate residues 90 min after synthesis was determined using an Ambis radioanalytical imaging system, and are expressed as % of the value obtained with WT HN analyzed in parallel. Values are the average of at least two experiments, ±10%.

The mutants described in this study are targeted to the ER and Ins N2, Del C1, and Del C2-4 are defective in transport from the ER, indicating that the folding of the S/A in the assembly of two HN monomers into a dimer. The finding that mutants Del N2 and Del N3 both sedimented as dimers (panels C and D) and yet displayed very different ER-to-Golgi transport phenotypes indicates that the efficiency with which a S/A mutant is transported from the ER does not strictly correlate the assembly of two dimers into a stable tetramer.

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| Assembly and transport of HN S/A mutants | WT | Del N1 | Del N2 | Del N3 | Del N4 | Ins N1 | Ins N2 | Ins N3 | Ins N4 | Del C1 | Del C2-4 |
|----------------------------------------|----|--------|--------|--------|--------|--------|--------|--------|--------|--------|----------|
| HN mutant                              |    |        |        |        |        |        |        |        |        |        |          |
| ER                                     |    |        |        |        |        |        |        |        |        |        |          |
| targeting                              |    |        |        |        |        |        |        |        |        |        |          |
| mAb reactivity                         |    |        |        |        |        |        |        |        |        |        |          |
| mAb reactivity                         |    |        |        |        |        |        |        |        |        |        |          |
| Oligomer                               |    |        |        |        |        |        |        |        |        |        |          |
| Oligomer                               |    |        |        |        |        |        |        |        |        |        |          |
| % of WT endoH resistant                |    |        |        |        |        |        |        |        |        |        |          |
| % of WT endoH resistant                |    |        |        |        |        |        |        |        |        |        |          |
| Cell surface                           |    |        |        |        |        |        |        |        |        |        |          |
| surface                                |    |        |        |        |        |        |        |        |        |        |          |
|                                         |    |        |        |        |        |        |        |        |        |        |          |
|                                         |    |        |        |        |        |        |        |        |        |        |          |
|                                         |    |        |        |        |        |        |        |        |        |        |          |
|                                         |    |        |        |        |        |        |        |        |        |        |          |
|                                         |    |        |        |        |        |        |        |        |        |        |          |

* Reactivity with HN conformation-specific mAb 4b, 60 min after synthesis.

* Oligomeric form determined by sucrose density gradient sedimentation: T, tetramer; D, Dimer; SD, soluble and secreted dimer; ND, not determined.

* The fraction of HN containing endoH-resistant carbohydrate residues 90 min after synthesis was determined using an Ambis radioanalytical imaging system, and are expressed as % of the value obtained with WT HN analyzed in parallel. Values are the average of at least two experiments, ±10%.

* Cell surface immunofluorescence staining with HN-specific mAb 4b. Fraction calculated as endoH resistant over fully glycosylated form.
tants (Del C1–C4) were radiolabeled with Tran35S-label for 30 min and incubated in chase medium for 90 min before analysis by SDS-PAGE. A, endoH analysis of cell-associated HN proteins. Following radiolabeling and a 90-min incubation in chase medium, cells were lysed and proteins were immunoprecipitated from the extracts with anti-HN serum. Immune complexes were incubated in the presence (+) or absence (−) of endoH before analysis by SDS-PAGE. B, secretion of COOH-terminal deletion mutants. Following a 3-h chase period, samples representing all of the medium (medium lanes) or one-fourth of the cell-associated protein (cell lanes) were immunoprecipitated with anti-HN serum and analyzed by SDS-PAGE.

FIG. 8. EndoH analysis and secretion of COOH-terminal S/A deletion mutants. VTF7.3-infected HeLa T4 cells were transfected with plasmid DNA encoding WT HN or one of the COOH-terminal deletion mutants Del C1–4 (lanes 1–4, respectively) and radiolabeled for 30 min with Tran35S-label. A, endoH analysis of cell-associated HN proteins. Following radiolabeling and a 90-min incubation in chase medium, cells were lysed and proteins were immunoprecipitated from the extracts with anti-HN serum. Immune complexes were incubated in the presence (+) or absence (−) of endoH before analysis by SDS-PAGE. B, secretion of COOH-terminal deletion mutants. Following a 3-h chase period, samples representing all of the medium (medium lanes) or one-fourth of the cell-associated protein (cell lanes) were immunoprecipitated with anti-HN serum and analyzed by SDS-PAGE.

Previous work on type I integral membrane proteins has shown that changes in the length of a membrane anchor do not always disrupt stop-transfer function or intracellular transport. For example, the COOH-terminal membrane anchor of the vesicular stomatitis virus G protein can be shortened from 20 to 14 residues without affecting anchoring or transport of the protein from the ER to the cell surface (Adams and Rose, 1985). For type II proteins, the length requirements for each of the S/A functions in biosynthesis and transport have not been extensively characterized. The in vivo data presented here indicate that changes in S/A length can have two general consequences on a biosynthetic step: a phase-specific effect for entry into and exit from the ER or a general effect on the structure of the ectodomain as seen for subunit assembly and carbohydrate processing. Increasing or decreasing the length of the hydrophobic core of the S/A by two residues resulted in polypeptides that displayed aberrant targeting to or translocation across the ER membrane. This phenotype did not simply reflect upper or lower size limitations on a functional HN S/A, because normal signaling function could be restored by further alterations involving greater than two residues. It is unlikely that this phenotype results from disruption of a signal in the primary amino acid sequence per se, since NH2-terminal S/A substitutions that do not change the length of the hydrophobic domain show no detectable defect in these steps (Parks and Pohlmann, 1995). Previous in vitro studies have led to the proposal that hydrophobicity is the dominant feature of a functional S/A (Spiess and Handschin, 1987; Zerial et al., 1987; reviewed in Nayak and Jabbour, 1989). In the case of cleavable signal sequences, theoretical considerations (reviewed in Engelman et al., 1986), as well as results from mutational and biophysical studies, have suggested that an additional critical feature of a functional signal sequence may be the propensity of the hydrophobic core to form an α-helical conformation (reviewed in Izard and Kendall, 1994). The data presented here indicate that an optimum length of the hydrophobic core of a S/A can greatly influence the efficiency with which a type II protein is properly targeted to the ER in vivo, and suggest that there is a functional requirement in the S/A along one face of an α-helix. While alterations of two residues could prevent the formation of an α-helical conformation in the nascent chain, it is also possible that these alterations change the relative position of critical residues along one face of an α-helix.

To determine if the HN COOH-terminal S/A deletion mutants were secreted from cells, infected/transfected cells were pulse-labeled with Tran35S-label for 30 min and incubated in chase medium for 3 h before analysis of cell-associated proteins and media. HN protein was detected in extracellular media from cells expressing mutants Del C2–C4, but not from cells expressing WT HN or mutant Del C1 (Fig. 8B). The lack of intracellular Del C2–C4 protein with endoH-resistant glycans (Fig. 8A) is consistent with the proposal that ER-to-Golgi transport of HN is rate-limiting, but once HN reaches the Golgi the transport to the cell surface (i.e. secretion) is very rapid (Parks and Lamb, 1990b). Thus, it appears that Del C2–C4 proteins are synthesized in a soluble and secreted form due to S/A deletions. Similar results have been reported previously for S/A deletion mutants of the influenza A virus NA and the MHC-associated invariant chain (Hogue and Nayak, 1994; Lipp and Dobberstein, 1986).

**DISCUSSION**

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Once inserted into the ER membrane, changes in the length of a transmembrane domain could have several possible consequences on S/A-lipid interactions: membrane-flanking sequences could be pulled into or could extend out of the lipid bilayer, the hydrophobic segment could adopt a compacted or extended structure, or the lipid bilayer itself could adapt to accommodate length changes in the hydrophobic segment of the polypeptide (Adams and Rose, 1985). Theoretical models (e.g. Mouritsen and Bloom, 1984) as well as experiments with bacteriorhodopsin in synthetic bilayers (Lewis and Engelman, 1983) suggest that it is more likely that changes will take place in the lipid bilayer to accommodate the length of the hydropho-
bic anchor domain than to have changes occur in the structure of the membrane-imbedded polypeptide chain. These putative changes in the thickness of the bilayer induced by the polypeptide chain could prevent transport from the ER to the Golgi, transport of HN could be restored by a further deletion that resulted in the removal of a total of three consecutive S/A residues. Assuming the lipid bilayer changes to accommodate S/A length, the efficient transport of this mutant from the ER is not consistent with a control mechanism that is based on partitioning of membrane proteins by lipid bilayer thickness alone. The easiest interpretation of these results is that HN S/A length is less important for efficient ER-to-Golgi transport than is overall conformation, and that the deletion of three residues restores a transport requirement along one face of an α-helix.

Several lines of evidence are consistent with a requirement for a critical structure along one face of a TM α-helix. First, sequence comparisons between the TM domains of various strains of the influenza virus HA (Lazarovits et al. 1990) and Newcastle disease virus HN proteins (Sakaguchi et al., 1989) have shown conservation of sequence at intervals that cycle every fourth or seventh position within the TM domain, respectively. Second, extensive mutagenesis of the TM domain of M13 coat protein and of glycoprin A has identified positions that are sensitive to assembly-disrupting mutations. These positions occur with a periodicity of ~4 residues, suggesting that these critical TM residues might compose a face of an α-helix that is involved in subunit interactions (Deber et al., 1993; Lemmon et al., 1992). In the case of the ε subunit of CD3, ER retention and degradation can be affected by changes in TM domain length, consistent with a requirement for correct positioning of a charged residue along a face of a TM α-helix (Lankford et al., 1993). Finally, the structure of the photosynthetic reaction center TM segments shows that residues forming helix-helix contact points are spaced at intervals of ~4 residues (Rees et al., 1989).

It is possible that the phase-sensitive transport defect seen with the HN S/A deletion mutants results from a defect in proper subunit helix-helix interactions in the lipid bilayer. Assuming the HN S/A exists as an α-helix in the membrane, the removal of three S/A residues might restore the correct face of an α-helix relative to neighboring subunits and allow transport whereas other alterations might prevent proper subunit interactions. However, it is important to note that HN S/A mutants with very different ER-to-Golgi transport characteristics were all dimeric, indicating that a defect in tetramer assembly does not always correlate with reduced transport from the ER (Parks and Pohlmann, 1995). A limitation of the sedimentation assay is the inability to distinguish between two types of assembly-defective mutants: those that have matured only to the point of forming dimers and those that sediment as dimers as a result of unstable tetramers which have dissociated during centrifugation (Parks and Pohlmann, 1995). Thus, all dimers detected by sedimentation analysis may not be equivalent, having apparent differences in assembly that account for the varying transport phenotypes.

For a subset of insertion mutants Ins N3 and Ins N4, only one of the four COOH-terminal carbohydrate residues was processed to an endoH-resistant form by Golgi-specific enzymes, contrasting with the two processed chains seen for WT HN (Ng et al., 1989). The link between processing of N-linked oligosaccharides and transport is not absolute, as examples of surface-expressed membrane proteins with endoH-sensitive N-linked carbohydrates have been described (Williams and Lamb, 1988). Furthermore, heterogeneity in carbohydrate processing may occur when membrane proteins are expressed in a soluble form in cDNA plasmids (e.g. influenza NA protein, Paterson and Lamb (1990) or may be a natural feature of some membrane proteins (Hubbard, 1988). Oligosaccharide processing can be influenced by local secondary structure around an N-linked carbohydrate site or by subunit interactions by a mechanism that may involve stearic inaccessibility (Ashford et al., 1993; Hubbard, 1988). Thus, the differential processing of Ins N3 and Ins N4 oligosaccharides can be viewed as indicative of changes in the ectodomain conformation or subunit interactions that may influence access to one of the COOH-terminal carbohydrate chains.

In summary, these data demonstrate that changes in S/A length can differentially affect various steps in the biosynthesis and transport of a type II protein. Entry into and exit from the ER are sensitive to S/A length changes in a phase-specific manner, suggesting a requirement in the S/A along one side of an α-helical configuration to function efficiently in these steps. The positioning of subunits may be a critical step during the formation of a disulfide-linked dimer or during interactions with cellular proteins involved in promoting assembly and intracellular transport.

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Diffenential Effects of Changes in the Length of a Signal/Anchor Domain on Membrane Insertion, Subunit Assembly, and Intracellular Transport of a Type II Integral Membrane Protein
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