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Aberrant Membrane Insertion of a Cytoplasmic Tail Deletion Mutant of the Hemagglutinin-Neuraminidase Glycoprotein of Newcastle Disease Virus

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The hemagglutinin-neuraminidase (HN) protein of Newcastle disease virus (NDV) is a type II glycoprotein oriented in the plasma membrane with its amino terminus in the cytoplasm and its carboxy terminus external to the cell. We have previously shown that the membrane insertion of HN protein requires signal recognition particle SRP, occurs cotranslationally, and utilizes the same GTP-dependent step that has been described for secretory proteins, type I proteins, and multispanning proteins (C. Wilson, R. Gilmore, and T. Morrison, Mol. Cell. Biol. 7:1386–1392, 1987; C. Wilson, T. Connolly, T. Morrison, and R. Gilmore, J. Cell Biol. 107:69–77, 1988). The role of the amino-terminal cytoplasmic domain in the faithful membrane insertion of this type II protein was explored by characterizing the membrane integration of a mutant lacking 23 of the 26 amino acids of the cytoplasmic domain. The mutant protein was able to interact with SRP, resulting in translation inhibition, membrane targeting, and membrane translocation, but the efficiency of translocation was considerably lower than for the wild-type HN protein. In addition, a significant proportion of the mutant protein synthesized in the presence of SRP and microsomal membranes was associated with the membrane in an EDTA- and alkali-insensitive manner yet integrated into membranes with its carboxy-terminal domain on the cytoplasmic side of membrane vesicles. Membrane-integrated molecules with this reverse orientation were not detected when the mutant protein was synthesized in the absence of SRP or a functional SRP receptor. Truncated mRNAs encoding amino-terminal segments of the wild-type and mutant proteins were translated to prepare ribosomes bearing arrested nascent chains. The arrested mutant nascent chain, in contrast to the wild-type nascent chain, was also able to insert into membranes in a GTP- and SRP-independent manner. Results suggest that the cytoplasmic domain plays a role in the proper membrane insertion of this type II glycoprotein.

Proteins that span the plasma membrane once have been classified into two categories, type I and type II, reflecting their final orientation within the membrane. Both types of proteins utilize the same signal recognition particle (SRP)-dependent mechanism for translocation across the rough endoplasmic reticulum (RER) membrane, a process that was first characterized for secretory proteins (for a review, see reference 31). SRP-dependent insertion initiates when the signal sequence of the nascent polypeptide chain emerges from the ribosome and is bound by the signal sequence recognition domain of SRP (24, 32). Concurrently, translation of the nascent polypeptide is inhibited by the translation arrest domain of SRP (24, 29, 30). The SRP-nascent chain-ribosome complex is then targeted to the RER membrane via the interaction between the translocation promotion domain of SRP (24) and the SRP receptor (docking protein) on the RER membrane (7, 8, 9, 20). More recently, GTP-dependent binding of the nascent chain to membranes has been demonstrated as a step preceding translocation (3) that most likely represents binding of SRP to its receptor and the concurrent release of the nascent chain from SRP (4). The GTP requirement for translocation has been observed for secretory as well as multispanning and single-spanning membrane proteins of both types (3, 13, 34).

Secretory proteins and type I membrane proteins both contain cleavable signal sequences (for a review, see reference 33). The mature amino terminus of type I proteins is in the lumen of the RER membrane, and the nascent chain is anchored in the membrane by a stop-transfer sequence located near the carboxy terminus. In contrast, type II membrane proteins contain a hydrophobic region near the amino terminus that functions as both a signal sequence and a membrane anchor (18, 26). The amino terminus of type II proteins remains on the cytoplasmic side of the RER membrane, whereas the carboxy terminus is translocated into the lumen. Although the membrane insertion of type II proteins is not clearly understood, the amino acid segments flanking the hydrophobic core of the bifunctional signal sequence influence type II membrane topology. Truncations of the amino-terminal flanking region can either cause cleavage by signal peptidase (16, 26) or reduce the efficiency of membrane insertion (1, 17, 25, 26). These flanking sequences may also play a role in orientation of the protein in the membrane (28).

The hemagglutinin-neuraminidase (HN) glycoprotein of Newcastle disease virus is a type II membrane protein. It contains a 26-amino-acid cytoplasmic tail, a 22-amino-acid hydrophobic signal-transmembrane region, and a 522-amino-acid translocated carboxy-terminal domain that contains six potential N-linked glycosylation sites (19). Our previous work demonstrated that HN protein is inserted into membranes cotranslationally in an SRP-dependent manner (35). To investigate the role of the amino-terminal flanking region in membrane insertion, we constructed a full-length HN cDNA clone and a mutant clone containing a truncated amino-terminal cytoplasmic tail. Removal of 23 of the 26

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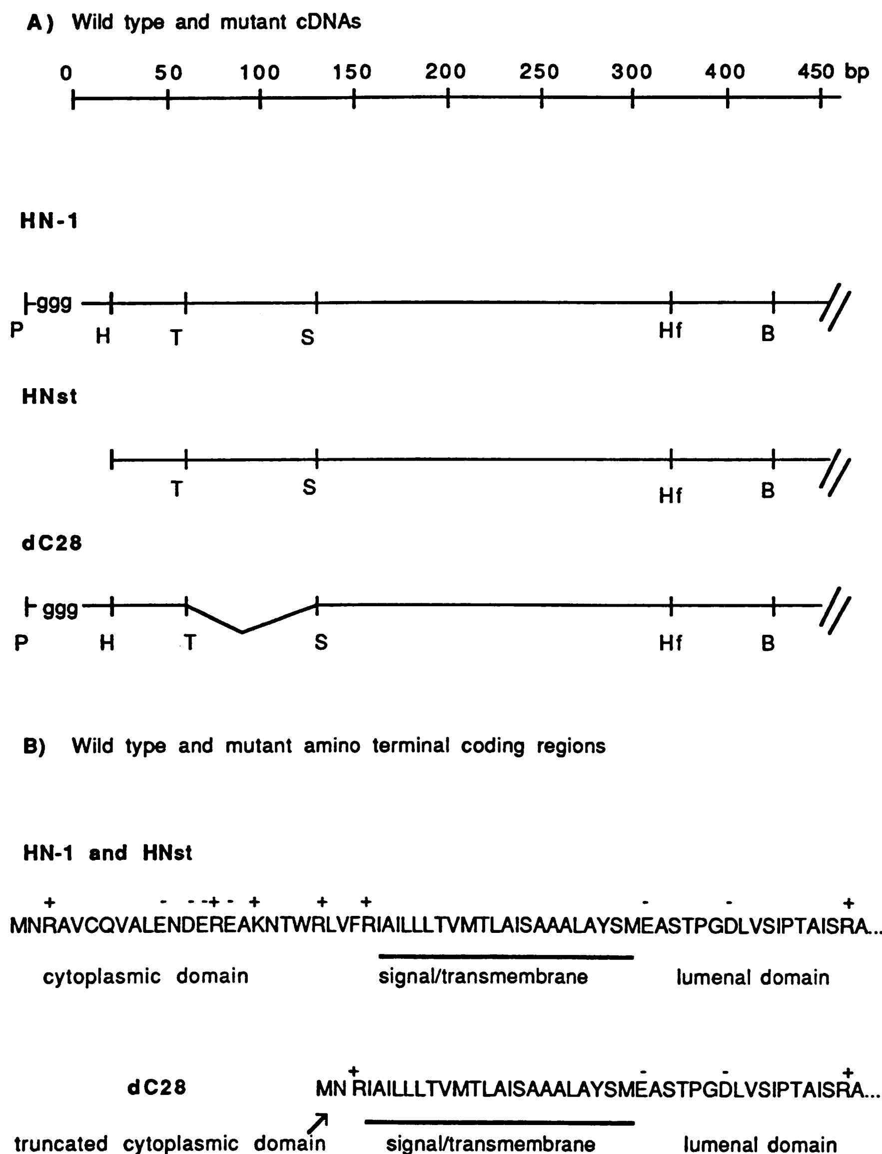

FIG. 1. Structures of the wild-type and mutant cDNAs and proteins. (A) Wild-type HN-1, HNst devoid of the 5′ GC tails, and the deletion mutant dC28, generated as described in Materials and Methods. Restriction enzyme sites: *Hph*I (H), *Tha*I (T), *Sau*3A (S), *Hin*fI (Hf), and *Bsp*1286 (B). (B) Predicted amino acid sequences for the amino-terminal regions for HN and dC28, shown in single-letter code. Symbols: +, positively charged residues; −, negatively charged residues; ▬, hydrophobic signal-transmembrane span.

amino acids of the cytoplasmic tail resulted in a decrease in insertion of the protein in the correct type II orientation. Instead, a significant proportion of the protein was inserted in a reverse orientation. Potential models for the role of flanking regions in membrane orientation are discussed.

MATERIALS AND METHODS

Construction of the cDNA clones in SP64. Plasmid HN-1, containing the entire coding region of HN, was constructed in pSP64 by ligation of three overlapping HN-containing cDNA clones, pTL2253, pTL709, and pTL119, as described previously (19). The three HN cDNA segments were joined at the *Cla*I site (base pair 855) and the *Pst*I site (base pair 1560) and inserted into pSP64 from the 5′ *Pst*I site in pTL2253 generated by the initial cloning procedure (10, 14) and the *Sac*I site in the 3′ untranslated region of pTL119. HNst, the plasmid devoid of the 5′ GC tails generated during cloning, was constructed by ligation of HN-1 DNA extending from the *Hph*I site at base pair 22 to the *Sac*I site at base pair 1872 into the *Sma*I and *Sac*I sites of pSP64 as described elsewhere (20a). The deletion mutant dC28 was generated by the in-frame ligation of HN-1 between the *Tha*I site at base pair 60 and a blunted *Sau*3A site at base pair 129, which was filled in with a Klenow reaction. The resulting cDNA is missing 23 of the 26 codons that encode the cytoplasmic tail of HN. Dideoxy-chain sequencing by the method of Sanger et al. (23) was done to verify the junction sequence. Structures of these plasmids are shown in Fig. 1A.

Transcription of full-length and truncated mRNA. Full-length transcripts for each clone were generated by cutting the template cDNAs with *Sac*I before transcription with SP6 RNA polymerase. The truncated transcripts for the mem-

brane-binding assays were generated by digesting the cDNA template with either *Hin*fI (resulting in mRNAs containing 68 codons of dC28 or 91 codons of HN-1) or *Bsp*1286 (resulting in mRNAs containing 99 codons of dC28 or 122 codons of HN-1).

Cell-free translation reactions. Wheat germ extract was prepared by the method of Erickson and Blobel (5). Wheat germ translation reaction mixtures contained 90 mM potassium acetate (KOAc), 2.5 mM $Mg(OAc)_2$, 1.9 mM ATP, 0.3 mM GTP, 33 mM creatine phosphate, 2.5 mM dithiothreitol (DTT), 0.16 mM amino acids, 63 μg of creatine phosphokinase per ml, 25 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), 0.08 mg of spermidine per ml, 15 μCi of $[^{35}S]$methionine (Dupont, NEN Research Products, Boston, Mass.), and 0.5 U of RNase inhibitor (Promega Biotech, Madison, Wis.) per 25 μl. Wheat germ translation reactions were incubated for 1 h at 25°C with full-length transcripts and for 15 min with the truncated transcripts.

Membrane-supplemented translation reactions. Salt-washed membranes (KRM) and SRP were isolated as described previously (32). *N*-Ethylmaleimide-treated KRM (N-KRM), which contain an inactivated SRP receptor, were prepared as described previously (8). A 1-μl sample of SRP (20 U) or membranes (1 equivalent) was added per 25 μl of translation reaction.

Incubation of polyribosomes with microsomal membranes. In vitro-assembled polyribosomes containing nascent HN or dC28 polypeptides were separated from ribonucleotides by gel filtration as described previously (3). Briefly, 100-μl samples of the translation reaction mixture were applied to a Sephacryl S-200 column equilibrated with 50 mM triethanolamine acetate (pH 7.5)–150 mM KOAc–2.5 mM $Mg(OAc)_2$–3 mM DTT–0.002% Nikkol. Nascent-chain insertion assays were conducted by incubating 25-μl samples of the void-volume fraction with 3 equivalents of KRM plus or minus 100 μM ribonucleotides for 10 min at 25°C. The samples were then EDTA extracted and separated into membrane pellet and supernatant fractions (see below).

Endoglycosidase H digestion. Samples (10 μl) of the translation reaction mixture were diluted in buffer to yield final concentrations of 1% sodium dodecyl sulfate (SDS), 0.15 M Tris base (pH 7.5), and 1% β-mercaptoethanol. The samples were boiled for 2 min, cooled to room temperature, and diluted to final concentrations of 0.25 M citric acid, 0.25 M sodium citrate, 0.01 M phenylmethylsulfonyl fluoride, 1% SDS, 0.15 M Tris base (pH 7.5), and 1% β-mercaptoethanol. The samples were then split and treated with either 1 μl of endoglycosidase H or citrate buffer containing 0.3 M citric acid and 0.3 M sodium citrate (pH 5.5). Samples were incubated at 37°C for 5 h and then precipitated with one-fifth volume of 100% trichloroacetic acid (TCA).

Trypsin protection. Samples (5 μl) of the translation reaction mixture were diluted in 20 μl of column buffer. The diluted samples were incubated with trypsin (final concentration, 100 μg/ml) for 30 min at 25°C. Protease digestions were terminated by adjustment to 200 μg of soybean trypsin inhibitor per ml. The digested samples were then precipitated with one-fifth volume of 100% TCA.

Membrane association assays. EDTA treatment of translation products (5-μl samples diluted with 20 μl of column buffer) or membrane-binding assay samples (30 μl) was accomplished by incubation for 10 min on ice in 25 mM EDTA. The samples were separated into membrane pellet and supernatant fractions by centrifugation in a step gradient containing a 50-μl cushion of 0.25 M sucrose–50 mM triethanolamine acetate–150 mM KOAc–25 mM EDTA–1 mM DTT for 5 min at 20 lb/in^2 in an airfuge (Beckman Instruments, Inc., Fullerton, Calif.) at 4°C. Alkali treatment of membranes was conducted by the addition of 70 μl of 0.1 M Na_2CO_3 (pH 11.5) to 5 μl of translation products. After 10 min, the alkali-treated sample was layered over a 60-μl cushion of 0.2 M sucrose [0.1 M Na_2CO_3, 150 mM KOAc, 2.5 mM $Mg(OAc)_2$, 1 mM DTT] and centrifuged for 7 min at 30 lb/in^2 in an airfuge. Supernatant fractions of each step gradient were precipitated by one-fifth volume of 100% TCA. Both the pellets obtained after TCA precipitation and the membrane pellets were solubilized in 10 μl of 0.5 M Tris base–6.25% SDS.

Polyacrylamide gel electrophoresis. Translation products (5 μl) or membrane supernatant fractions were TCA precipitated before being dissolved in 10 μl of 0.5 M Tris base–6.25% SDS. The samples were boiled in the presence of 0.1 M DTT for 5 min and alkylated by adjustment to 55 mg of iodoacetamide per ml. Full-length products were resolved on 10% polyacrylamide-SDS gels, and the truncated products were resolved on 12 to 20% gradient gels, followed by fluorography (15).

RESULTS

Construction of HN and mutant cDNAs. Full-length HN cDNA (HN-1) was assembled in pSP64 by ligation of three overlapping HN-specific clones. The GC tails produced during the original cloning protocol were removed from HN-1, resulting in HNst (Fig. 1A). RNA transcribed in vitro by SP6 RNA polymerase was used to program wheat germ extract translations in the presence and absence of SRP and KRM. The HN-1 and HNst translation products comigrated with the previously characterized glycosylated and unglycosylated HN protein synthesized in reactions programmed with NDV-infected cell mRNA (data not shown).

The mutant dC28 (Fig. 1A) was constructed by the in-frame ligation of HN-1 cDNA between base pairs 60 and 129, resulting in a cDNA missing 23 of the 26 codons that encode the cytoplasmic tail. The protein encoded by dC28 has a cytoplasmic tail of only three amino acids with a single positive charge (Fig. 1B), in contrast to the 26-amino-acid tail of the HN-1 protein, which contains five positive and four negative charges.

Translation of HN and dC28 mRNAs in the presence and absence of SRP and KRM. The wild-type and mutant mRNAs were translated in wheat germ cell-free translation reactions in the presence and absence of SRP and KRM to assess their interaction with SRP. Translation of HN-1 mRNA in the presence of SRP alone (Fig. 2A, lane 5) resulted in a 67% decrease in synthesis compared with translation in the absence of SRP (Fig. 2A, lane 4). This decrease is diagnostic of an SRP-mediated inhibition of translation. In the presence of both SRP and KRM, a significant amount of glycosylated HN was obtained upon translation of HN-1 mRNA (HNg; Fig. 2A, lane 6).

As expected, the unglycosylated dC28 product synthesized in the absence of SRP had a faster electrophoretic mobility than did unglycosylated HN because of the deletion of 23 amino acids (dC28ug; Fig. 2A, lane 1). The synthesis of dC28 protein decreased to roughly the same value (58%) as did HN (67%) in the presence of SRP alone (Fig. 2A, lane 2). The addition of KRM to translation reactions programmed with dC28 mRNA relieved the SRP arrest of elongation, resulting in an increase in synthesis of the dC28 polypeptide over that obtained in the presence of SRP alone. In addition, translation of dC28 mRNA in the presence of both SRP and

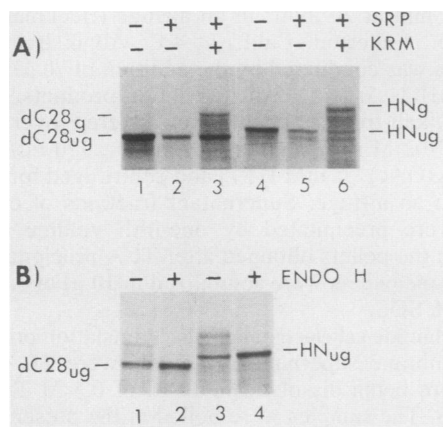

FIG. 2. Translation of wild-type and mutant mRNAs in the presence and absence of SRP and membranes. (A) Wheat germ translation reactions were programmed with mRNAs from dC28 (lanes 1 to 3) and HN-1 (lanes 4 to 6) in the absence of SRP and KRM (lanes 1 and 4), presence of SRP (lanes 2 and 5), or presence of both SRP and KRM (lanes 3 and 6). The unglycosylated (ug) and glycosylated (g) forms of each protein are labeled. (B) Translation products synthesized in the presence of both SRP and KRM were treated with endoglycosidase H (ENDO H; +) or citrate buffer (−), as indicated.

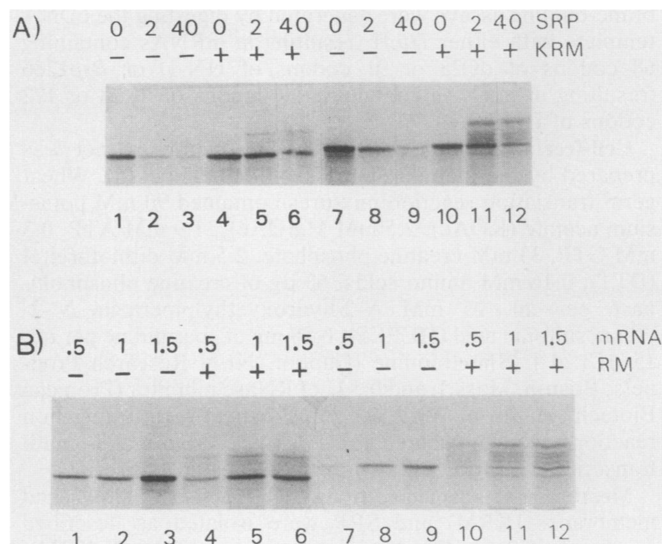

FIG. 3. Translation of wild-type and mutant mRNAs with increasing levels of SRP. (A) Translation mixtures (25 μl) of dC28 (lanes 1 to 6) and HN-1 (lanes 7 to 12) contained either 2 U (lanes 2, 5, 8, and 11) or 40 U (lanes 3, 6, 9, and 12) of SRP, no KRM (lanes 1 to 3 and 7 to 9), or KRM (lanes 4 to 6 and 10 to 12). (B) Varying amounts of RNA (0.5 μl [lanes 1, 4, 7, and 10], 1.0 μl [lanes 2, 5, 8, and 11], and 1.5 μl [lanes 3, 6, 9, and 12]) from dC28 (lanes 1 to 6) and HN-1 (lanes 7 to 12) transcription reactions were added to translation reactions with (lanes 4 to 6 and 10 to 12) and without (lanes 1 to 3 and 7 to 9) SRP and KRM.

KRM yielded a polypeptide that was likely glycosylated (dC28g; Fig. 2A, lane 3). However, there was consistently less glycosylated product in dC28 RNA-directed reactions than in wild-type RNA-directed reactions. Densitometric scanning of lanes 3 and 6 revealed that approximately 46% of the dC28 and 82% of the HN-1 products were glycosylated in the presence of both SRP and KRM. Table 1 shows results of this and duplicate experiments.

Endoglycosidase H digestion of HN and dC28 products. The HN protein is not processed by signal peptidase during synthesis and membrane integration. However, several cytoplasmic tail deletion mutants of type II proteins appear to be processed in the presence of membranes (16, 26). Signal sequence cleavage of glycosylated dC28 might conceivably result in synthesis of a polypeptide with a mobility identical to that of the unglycosylated precursor. Carbohydrate was

TABLE 1. SRP-mediated inhibition of synthesis and translocation of dC28 and HN-1[a]

Expt	SRP (U)	dC28		HN-1	
		% Inhibition[b]	% Glycosylation[c]	% Inhibition	% Glycosylation
1	20	58	46	67	82
2	20	ND[d]	42	ND	58
3	20	ND	38	ND	58
4	2	80	25	55	48
	40	89	40	87	70

[a] Densitometry was used to calculate the amount of unglycosylated dC28 and HN protein (any protein migrating at the expected position of unglycosylated dC28 or HN) and glycosylated protein (any protein migrating slower than the unglycosylated protein but no slower than the completely glycosylated dC28 or HN protein) in experiments 1 (Fig. 2A), 2 (Fig. 2B), 3 (Fig. 4A), and 4 (Fig. 3A, lanes 1 and 7).

[b] Calculated as [1 − (amount of protein produced in the presence of SRP/amount of protein produced in the absence of SRP)] × 100.

[c] Calculated as (amount of glycosylated protein/amount of unglycosylated plus glycosylated protein) × 100.

[d] ND, Not done.

removed from dC28 translation products by endoglycosidase H digestion to determine whether any of the translocated protein had undergone cleavage by signal peptidase. Signal sequence removal followed by endoglycosidase H treatment should result in a product that migrates faster than the unglycosylated dC28 or HN precursor. Endoglycosidase H digestion of the HN and dC28 translation products did not produce polypeptides that migrated more rapidly than the unglycosylated precursors (Fig. 2B; compare lanes 2 and 4 and lanes 1 and 3). Therefore, there was no detectable cleavage of either product by signal peptidase.

Translation of HN and dC28 mRNA at varying mRNA/SRP ratios. To determine whether a suboptimal concentration of SRP was responsible for the decreased glycosylation of the mutant protein, the syntheses of dC28 and HN protein were compared in reactions containing varying amounts of SRP. The total yield of glycosylated products was constant in both dC28 and HN mRNA-directed reactions (Fig. 3A, lanes 5 and 6 and lanes 11 and 12, respectively) when the SRP concentration varied over a 20-fold range. The percentage of the glycosylated product increased at the higher SRP concentration as a result of increased SRP inhibition of translation. Similarly, a decrease in the amount of mRNA added to the translation reaction did not lead to a greater efficiency of dC28 glycosylation (Fig. 3B, lanes 4 to 6). Therefore, SRP did not appear to be limiting component in these experiments. A consistently lower percentage of dC28 products than wild-type products became glycosylated in experiments in which KRM-mediated release of elongation arrest was clearly demonstrated (Fig. 2A and 3A). In addition, under experimental conditions that resulted in synthesis of similar levels of dC28 and HN protein (Fig. 3B; compare lanes 1 and 9), dC28 was not glycosylated to the same extent as the wild-type protein (Fig. 3B; compare lanes 4 and 12). Al-

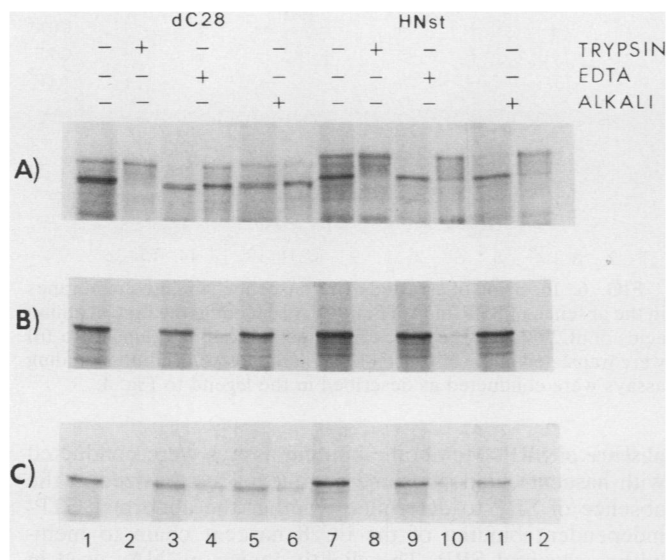

FIG. 4. Membrane topologies of the mutant and wild-type proteins. Samples were synthesized in the presence of SRP and KRM (A), N-KRM alone (B), and SRP and N-KRM (C). Total translation products (lanes 1 and 7) were subjected to trypsin digestion (lanes 2 and 8), EDTA extraction and fractionation (lanes 3, 4, 9, and 10), or alkali extraction and fractionation (lanes 5, 6, 11, and 12). Supernatant fractions are in the odd-numbered lanes, and membrane pellet fractions are in the even-numbered lanes.

though SRP inhibition and KRM-induced release of inhibition were detected for the mutant, these events led to inefficient translocation, as assayed by glycosylation, relative to the wild-type protein. The extent of SRP inhibition and protein glycosylation for dC28 and HN protein is shown in Table 1.

Membrane topology of HN and dC28 products. Trypsin protection experiments were performed to define the membrane orientation of the glycosylated and unglycosylated polypeptides. Glycosylated HN was protected from trypsin, since the bulk of the molecule had been translocated into the lumen of vesicles (Fig. 4A, lane 8). The unglycosylated HN precursor remained trypsin sensitive, since it is external to membranes (Fig. 4A, lane 8). Similarly, the glycosylated dC28 protein was protected from digestion, and the unglycosylated dC28 protein was digested (Fig. 4A, lane 2). Therefore, the bulk of dC28 protein synthesized in the presence of SRP and KRM was indeed nontranslocated, since it was nonglycosylated and trypsin sensitive.

EDTA and alkaline extraction experiments were conducted to determine whether the dC28 protein was stably associated with the membrane after synthesis. EDTA extraction removes peripheral proteins from the pelletable membrane fraction, whereas alkaline pH treatment removes all secretory and nonintegral membrane proteins from the membrane (6, 27). dC28 and HN-1 products synthesized in the presence of SRP and KRM were separated into membrane pellet and supernatant fractions after EDTA or alkali extraction. As expected, 25 mM EDTA did not extract glycosylated HN from the membrane but did extract majority of the the nontranslocated, unglycosylated form (Fig. 4A, lanes 9 and 10). The glycosylated dC28 protein was also not extracted from membranes by EDTA, since it, like the wild-type protein, is translocated across the membrane (Fig. 4A, lanes 3 and 4). In distinct contrast to the wild-type protein, over half of the unglycosylated dC28 protein remained associated with membranes after EDTA treatment (Fig. 4A, lanes 3 and 4). In addition, the percentages of the total products (glycosylated and unglycosylated) that were membrane associated were similar for dC28 and the wild-type HN.

The more stringent alkaline pH extraction removed the majority of the nonglycosylated HN protein but did not extract significant amounts of the glycosylated HN protein (Fig. 4A, lanes 11 and 12), demonstrating that the stable integration of only the glycosylated HN had occurred. Alkali treatment of dC28 translation reactions resulted in partial extraction of the glycosylated species (Fig. 4A, lanes 5 and 6), suggesting that the translocated dC28 product was not as stably associated with the membranes as was the wild-type product. Surprisingly, a large proportion of the unglycosylated dC28 product was remarkably resistant to alkali extraction. The presence of unglycosylated dC28 product in the membrane pellet fraction after alkaline extraction was not due to aggregation (Fig. 4B). Although dC28 was able to become translocated, glycosylated, and membrane anchored, a significant proportion of the dC28 synthesized in the presence of SRP and KRM was unglycosylated and trypsin sensitive yet integrated into membranes. Thus, dC28 may bury its amino terminus in the membrane without undergoing translocation.

Analysis of the SRP and SRP receptor dependency of dC28 insertion. The data suggest that transport of dC28 protein across membranes is less efficient than transport of wild-type HN protein. Conceivably, membrane association of the unglycosylated dC28 protein could occur by an SRP-independent insertion mechanism. To test this hypothesis, translation reactions were performed in the presence of N-KRM. The alkylated membranes were used to ensure that SRP-mediated targeting could not occur, since *N*-ethylmaleimide treatment inactivates both SRP (29) and the SRP receptor (9). Neither dC28 nor HN protein became trypsin protected when N-KRM were present (Fig. 4B, lanes 2 and 8). Significantly, very little dC28 or wild-type HN protein remained in the membrane pellet fraction after either EDTA or alkali extraction procedures (Fig. 4B, lanes 3 and 6 and lanes 9 and 12). The quantity of dC28 protein recovered in the pellet fraction was similar to that obtained in the absence of membranes and presumably represents aggregated protein (data not shown). Similar results were obtained when dC28 mRNA was translated in the presence of SRP and N-KRM (Fig. 4C). Therefore, membrane association of both the glycosylated dC28 and the reverse-oriented dC28 requires both SRP and the SRP receptor.

Binding of nascent chains to membranes in the presence of SRP. The results presented above show that the interaction of dC28 protein with SRP results in elongation arrest and membrane targeting. Yet integration of dC28 has occurred in an abnormal fashion, with a substantial proportion of the polypeptide inserted into the membrane in an orientation opposite that of the wild-type HN protein. Since SRP-mediated targeting occurs with nascent chain-ribosome complexes, we investigated the early events in membrane targeting of the dC28 protein to determine whether there was a detectable abnormality at this stage.

Like secretory, type I, and multispanning proteins, the HN nascent chain undergoes GTP-dependent membrane insertion preceding translocation (34). To determine whether this step in membrane integration of dC28 protein was abnormal, truncated mRNAs encoding the NH_2-terminal portions of dC28 (68 residues) and HN-1 (91 residues) were translated in the presence of SRP. The SRP-ribosome-

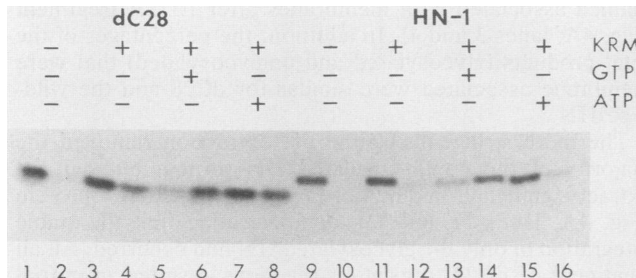

FIG. 5. Insertion of arrested dC28 nascent chain in the absence of GTP and in the presence of SRP. mRNAs encoding the first 68 amino acids of dC28 or 91 amino acids of HN were translated in the presence of SRP for 15 min in a 100-μl wheat germ translation mixture. After the addition of emetine, the nascent chain-ribosome complexes containing dC28-68 (lanes 1 to 8) and HN-91 (lanes 9 to 16) were isolated from a Sephacryl S-200 column, incubated for 10 min at 25°C in the presence or absence of KRM, GTP, or ATP (as indicated), treated with 25 mM EDTA for 10 min on ice, and fractionated into supernatant (odd-numbered lanes) and membrane pellet (even-numbered lanes) via airfuge centrifugation over a 0.25 M sucrose cushion.

nascent chain complexes were isolated, and the membrane insertion of each nascent chain was assayed as described previously (3, 22). In the absence of ATP and GTP, the HN nascent chains were readily extracted from membranes when the ribosomes were disrupted with EDTA (Fig. 5, lanes 11 and 12; results are quantitated in Table 2). The majority of HN nascent chains fractionated in the membrane pellet when GTP was present during the membrane-binding reaction (Fig. 5, lanes 13 and 14). In contrast, dC28 nascent chains showed significant binding (33%) to membranes in the absence of ribonucleotide triphosphates (Fig. 5, lanes 3 and 4). However, the percentage of the dC28 nascent chains that fractionated in the membrane pellet was dramatically increased (86%) in the presence of GTP (Fig. 5, lanes 5 and 6). Although dC28 nascent chains bound to membranes in the presence of ATP (Fig. 5, lanes 7 and 8), the amount bound probably reflects the additive effect of the ribonucleotide-independent binding (Fig. 5, lanes 3 and 4) and a very slight stimulation of binding by ATP that was also observed with the HN nascent chain (Fig. 5, lanes 15 and 16).

Binding of dC28 nascent chains to membranes in the absence of SRP. Membrane-binding assays were conducted with nascent chain-ribosome complexes synthesized in the absence of SRP to determine whether the abnormal GTP-independent binding of the dC28 nascent chain to membranes required SRP. The slightly longer mRNAs used in this assay encoded 99 and 122 amino acids of the dC28 and HN proteins, respectively (Table 2). Translation of the longer mRNAs results in the synthesis of a set of three polypeptides. The largest polypeptide corresponds to the full-length translation product of the truncated mRNA, whereas the two smaller polypeptides probably arise from internal pause sites or ribosome stacking (36). The HN nascent chain was unable to bind to the membrane under any of the conditions tested when SRP was not present (Fig. 6, lanes 9 to 16). However, in the absence of SRP, a significant proportion of the dC28 nascent chains bound to the membrane in the absence of GTP (41%; Fig. 6, lanes 3 and 4; results are quantitated in Table 2). The smaller translation products preferentially fractionated into the pellet fraction. The SRP-independent insertion of dC28 nascent chains was not stimulated by the addition of GTP (Fig. 6, lanes 5 and 6). Therefore, GTP stimulation of membrane binding for both HN and dC28 requires SRP. Identical results were obtained with N-KRM (not shown). Truncated dC28 nascent chains could be inserted by a mechanism that requires neither SRP, SRP receptor, nor GTP. Thus, failure to observe reverse-oriented insertion of full-length dC28 in the absence of SRP was not likely due to inactivation of an *N*-ethylmaleimide-sensitive receptor distal to the SRP receptor described by Nicchitta and Blobel (21).

TABLE 2. Binding of dC28 and HN-1 nascent chains to membranes[a]

Clone	Nascent chain length (amino acids)	SRP	% of nascent chain in pellet under given assay conditions[b]					
			No addition	KRM	KRM + GTP	KRM + ATP	Stimulation[c] GTP	Stimulation[c] ATP
HN-1	91	+	0	2	82	8	80	6
dC28	68	+	1	33	86	40	53	7
dC28	99	+	2	44	86	57	42	13
HNst	122	−	0	9	8	7	0	0
dC28	68	−	10	60	68	64	8	4
dC28	99	−	1	41	45	50	4	9

[a] Scanning densitometry was used to calculate the percentage of total products in the membrane pellet. Translation of the entire truncated mRNA would yield polypeptide of the indicated lengths.

[b] Calculated as [amount of protein in pellet fraction/(amount of protein in pellet fraction + amount of protein in supernatant fraction)] × 100.

[c] Calculated as percentage of nascent chain in the pellet fraction in the presence of GTP or ATP minus the percentage in the absence of ribonucleotide triphosphates.

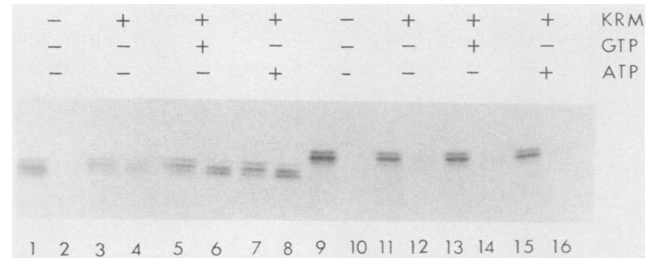

FIG. 6. Insertion of arrested dC28 nascent chain into membranes in the absence of SRP and GTP. mRNAs encoding the first 99 amino acids of dC28 (lanes 1 to 8) or 122 amino acids of HN (lanes 9 to 16) were translated in the absence of SRP, and posttranslational binding assays were conducted as described in the legend to Fig. 4.

DISCUSSION

The deletion mutant dC28, which lacks 23 of the 26 codons encoding the amino-terminal cytoplasmic tail of HN protein, directs synthesis of a protein that is able to interact with SRP, as shown not only by SRP-mediated translation arrest but also by the synthesis of glycosylated dC28 in an SRP-dependent manner. However, the luminal domain of dC28 polypeptide is less efficiently transported across the membrane than is wild-type HN. The majority of the dC28 proteins synthesized in the presence of SRP and KRM remained unglycosylated and trypsin sensitive. However, more than half of the unglycosylated polypeptides appear to be integrated into the membrane, since they resist extraction by EDTA or alkaline pH. These characteristics are compatible with a reversed orientation of dC28 in the membrane. An SRP-SRP receptor-dependent mechanism is involved in the insertion reaction, since only trace amounts of the full-length dC28 protein sediment with the membrane fraction in the

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                                                                                          MEMBRANE
                                                                                          ASSOCIATION
HN-1            +       - --+- +   +   +                             -  ..-    .   + -.
        MNRAVCQVALENDEREAKNTWRLVFRIAILLLTVMTLAISAAALAYSMEASTPGDLVSIPTAISRAEG           II
dC28                               +                                 -  ..-    .   + -.
                                MNRIAILLLTVMTLAISAAALAYSMEASTPGDLVSIPTAISRAEG      wk II, II*

Iγcat    -- +-     -  .  .++.. .- +  +.   .            .            .+ -+
     MDDQRDLISNNEQLPMLGRRPGAPESKCSRGALYTGFSILVTLLLAGQATTAYFLYQQQGRLDKLTVTSQNLQL    II
ΔNIγcat                                 .     .          .          .+ -+
(Lipp and Dobberstein, 1986)        MGALYTGFSILVTLLLAGQATTAYFLYQQQGRLDKLTVTSQNLQL  clv

ASGP
 +-  -  + - -- -++  ++..... .  +   ..+         .             .          -- +. +-
 MTKEYQDLQHLDNEESDHHQLRKGPPPPQPLLQRLCSGPRLLLLSLGLSLLLLVVVCVIGSQNSQLQEELRGLRET     II
pSA1-N                              ..+        .             .          -- +. +
(Spiess and Lodish, 1986)           MGPRLLLLSLGLSLLLLVVVCVIGSQNSQLQEELRGLRET       II, clv^a

NA                              . +    .      .       .       +    .   + .
                              MNPNQKIITIGSICMVVGIISLILQGNIISIWISHSIQTGNQNHTGICN    II
N15                                       .       .       +    .   + .
                                         MVVGIISLILQGNIISIWISHSIQTGNQNHTGICN       wk II
N18                                     . .+-.      .     +    .   + .
(Brown et al., 1988)                    MPAGRDGIISLILQGNIISIWISHSIQTGNQNHTGICN     wk II

pc2P (P-450 pro.P(N)TH)                 -   .           +  +.+ -.+  +++
                                        MDLVVVLGLCLSCLLLLSLWKQSHGRSDGKSVKKR        II*
pc2P1                                   ++  .           +  +.+ -.+  +++
(Szczesna-Skorupa et al., 1987)         MKRVVVLGLCLSCLLLLSLWKQSHGRSDGKSVKKR        II, clv
```

FIG. 7. Comparison of the amino termini of several wild-type and mutant proteins and their respective membrane associations. Symbols above the amino acids refer to charge (+ or −) or helix breakers (.). The hydrophobic region of each set of proteins is underlined. The membrane associations are listed according to the authors' characterization by the criterion of glycosylation, protease protection, signal sequence cleavage, and membrane fractionation studies (low salt or alkaline extraction). Abbreviations and symbols: II, N terminus in cytoplasm, carboxy terminus in membrane; II*, N terminus in membrane, carboxy terminus in cytoplasm; clv, signal sequence cleavage; wk II, weakly anchored in the membrane. [a]Although membrane fractionation studies were not done, the authors suggest that pSA1-N was in a type II form and that a low level of signal peptide cleavage occurred.

absence of SRP or SRP receptor. Thus, the decrease in translocation efficiency of dC28 is not due to a failure to target via the SRP-mediated mechanism but is instead caused by an abnormality in the directionality of the membrane insertion reaction. These results clearly show that the amino-terminal cytoplasmic tail influences the efficiency of proper membrane insertion in this system.

The SRP and GTP dependence of membrane binding was analyzed in studies using ribosome-tethered wild-type and mutant nascent chains to determine whether there was a defect in the initiation of translocation. GTP stimulated membrane insertion of dC28 nascent chains in the presence of SRP. However, in the absence of either SRP or a functional SRP receptor, high levels of GTP-independent insertion were also detected with dC28 nascent chains. GTP did not stimulate this SRP-independent insertion. Therefore, the GTP-mediated stimulation of nascent chain membrane insertion requires SRP.

Experiments using truncated transcripts of the wild-type and mutant mRNAs clearly demonstrate that the amino terminus of dC28 is capable of abnormal GTP- and SRP-independent interactions with the membrane. Can the SRP-dependent reverse insertion of the full-length dC28 polypeptide be explained by the ability of the arrested nascent chain to insert in an SRP-independent manner? The results indicate that insertion of dC28 into the membrane in either orientation is SRP dependent and is preceded by SRP-mediated translation inhibition, membrane targeting, and KRM-mediated release of translation arrest. A ribosome complex containing dC28 mRNA, a dC28 nascent chain, and SRP will be targeted to the SRP receptor when enough of the dC28 signal sequence has emerged to allow SRP recognition. The initial SRP-nascent chain interaction may lead to translocation of the ectodomain of dC28 across the membrane. However, another fate for the nascent chain becomes possible when the dC28 mRNA is targeted to the membrane. dC28 nascent chains that are synthesized after the mRNA has been targeted to the membrane may be close enough to the lipid bilayer to undergo the spontaneous, GTP-independent insertion reaction observed under the artificial circumstance of an arrested translation using truncated mRNAs. The initial SRP-dependent insertion would result in glycosylated dC28 polypeptides, whereas subsequent SRP-independent insertion reactions would result in the incorrectly oriented polypeptides.

In the case of the HN nascent chain, it is clear that the wild-type amino terminus is incapable of a GTP-independent insertion into the membrane with or without SRP present. The long, hydrophilic cytoplasmic domain may prevent spontaneous insertion of the hydrophobic membrane-spanning region by preventing contact between the lipid bilayer and the signal sequence. Therefore, the amino-terminal flanking region of HN may help maintain the directionality of membrane insertion by favoring the interaction between the hydrophobic signal sequence and SRP while eliminating contact between the signal sequence and the hydrophobic lipid bilayer. By favoring interaction with SRP, the wild-type HN amino-terminal region ensures that all membrane-targeted HN nascent chains become type II membrane proteins.

Although our data are consistent with this model of reverse insertion, we cannot rule out other explanations for our data. For example, absence of a cytoplasmic tail may result in an amino terminus that is unable to maintain an interaction with the translocation machinery that leads to faithful translocation of the carboxy terminus. Thus, at a certain frequency, the amino terminus of dC28 may insert into a translocation pore in a reverse orientation. The hydrophobic signal sequence will then span the membrane in a linear form topologically equivalent to a stop-transfer sequence. Such a configuration would likely terminate translocation, thereby resulting in a nonglycosylated protein that is stably integrated into the membrane. Although these two models differ, we have not been able to discriminate between them on the basis of our current data.

There are several reports in the literature concerning the membrane topology of proteins with mutations in their amino-terminal flanking regions (Fig. 7). Clearly, removal of the cytoplasmic domain of various type II proteins results in a variety of outcomes (compare the first four sets of wild-type and mutant proteins in Fig. 7). Membrane-integrated proteins with a reverse orientation were not produced upon translation of any of the previous constructs. The last set of proteins (pc2P and pc2P1) demonstrates that conversion of the N-terminal negative charge of pc2P to two positive charges allows translocation of the carboxy-terminal flanking domain of the hydrophobic core and changes the membrane orientation of this protein. dC28 protein retains a single positive charge in its N-terminal region, as do pSA1-N and N18, yet unlike the case with other type II deletion mutants, the orientation of the membrane-associated dC28 protein is not faithfully maintained. There are several features of the dC28 nascent chain that may account for the different fate of the dC28 protein relative to that of the other NH_2-terminal mutants.

First, the transmembrane span of dC28 is the only hydrophobic core that has a predicted alpha-helical configuration in an aqueous environment (2). In addition, the hydrophobic segment of dC28 contains no helix-breaking residues, whereas the other proteins contain one to three helix breakers in the transmembrane span. Second, the carboxy-terminal flanking segment is less hydrophilic than the corresponding segment from the other proteins, and the distance between the hydrophobic core and the first positively charged amino acid is much greater for dC28 than for any of the other proteins.

It seems likely that the orientation and membrane association of integral proteins are dependent on the properties of the hydrophobic core as well as the amino and carboxyl flanking regions, as suggested by others (11, 12, 25). An alteration in one of these segments may induce several possible membrane topologies, depending on the properties of the other two segments. The precise role for hydrophobicity, hydrophilicity, and charge distribution in determining the orientation of a protein during translocation remains speculative at present. Hartman et al. (12) have recently proposed that the difference in the charges of the 15 residues flanking the signal-anchor sequence determines the orientation of the protein in membranes. Whereas the orientation of wild-type HN protein is consistent with this proposal, the orientation of the abnormally inserted forms of mutant dC28 is not. Perhaps site-directed mutagenesis of the hydrophobic region as well as the carboxyl flank of dC28 will shed light on the role of these features in orienting a protein for translocation.

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