Translation and Membrane Insertion of the Hemagglutinin-Neuraminidase Glycoprotein of Newcastle Disease Virus

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The hemagglutinin-neuraminidase (HN) protein of paramyxoviruses is likely in the unusual class of glycoproteins with the amino terminus cytoplasmic and the carboxy terminus lumenal or external to the cell. The properties of the membrane insertion of the HN protein of Newcastle disease virus, a prototype paramyxovirus, were explored in wheat germ extracts containing microsomal membranes. HN protein was inserted into membranes cotranslationally, resulting in a glycosylated protein completely resistant to trypsin and proteinase K digestion. No detectable posttranslational insertion occurred. Insertion required signal recognition particle. Signal recognition particle in the absence of membranes inhibited HN protein synthesis. Comparisons of the trypsin digestion products of the HN protein made in the cell-free system with newly synthesized HN protein from infected cells showed that the cell-free product was in a conformation different from that of the pulse-labeled protein in infected cells. First, trypsin digestion of intact membranes from infected cells reduced the size of the 74,000-dalton HN protein by approximately 1,000 daltons, whereas trypsin digestion of HN protein made in the cell-free system had no effect on the size of the protein. Second, trypsin digestion of Triton X-100-permeabilized membranes isolated from infected cells resulted in a 67,000-dalton trypsin-resistant HN protein fragment. A trypsin-resistant core of comparable size was not present in the digestion products of in-vitro-synthesized HN protein. Evidence is presented that the newly synthesized HN protein in infected cells contains intramolecular disulfide bonds not present in the cell-free product.

Most eucaryotic transmembrane proteins cotranslationally integrate into the membrane of the rough endoplasmic reticulum (42). The signal recognition particle (SRP) binds to nascent polypeptides with rough endoplasmic reticulum signal sequences, and the SRP receptor or docking protein together with SRP mediates binding of the ribosome to the endoplasmic reticulum (17, 19, 27, 38, 39, 40). As protein synthesis continues, transport of the protein across the membrane proceeds until a stop transfer sequence terminates translocation (42). Many plasma membrane glycoproteins studied have an amino-terminal signal sequence, which is usually cleaved, and a stop transfer sequence near the carboxy terminus (42). The stop transfer sequence serves as the membrane anchor. As initially integrated into the rough endoplasmic reticulum, these proteins are oriented with the amino terminus in the lumen and the carboxy terminus cytoplasmic.

A small class of transmembrane proteins are inserted in the opposite orientation. Most of these proteins, for example, the transferrin receptor (32), the HLA-DR invariant chain (37), the asialoglycoprotein receptor (22, 35), and the influenza neuraminidase glycoprotein (7), have no cleaved signal sequence. Rather, a sequence at or near the amino terminus may function as both a signal sequence and a transmembrane-spanning sequence (35). Not all membrane anchor sequences can function as signal sequences, even when placed at the amino terminus of a polypeptide (43). Thus, analysis of these atypical bifunctional signal sequences should facilitate description of sequence elements which contribute to each function. Analysis of amino-terminal membrane anchor sequences may also provide clues about elements which determine the membrane orientation of a protein.

Recent analyses of several different paramyxovirus hemagglutinin-neuraminidase (HN) glycoprotein genes have suggested that the HN protein may belong to this unusual class of proteins (1, 5, 13, 20, 41; L. McGinnes, A. Wilde, and T. Morrison, *Virus Res.*, in press). The predicted amino acid sequences of these HN proteins contain a short charged amino terminus (17 to 40 residues) followed by a hydrophobic stretch of 22 to 26 amino acids. No other potential membrane-spanning region, as defined by Kyte and Doolittle (23), exists in the sequences. On the basis of these predicted amino acid sequences, it has been suggested that paramyxovirus HN proteins are oriented with the amino terminus cytoplasmic and carboxy terminus external or lumenal (1, 5, 13, 20, 41; McGinnes et al., in press). Indeed, there is evidence that the paramyxovirus Newcastle disease virus (NDV) HN protein carboxy terminus is exposed on the external surface of the viral membrane (33). In addition, there is no evidence for a cleaved signal sequence (9, 10). Thus, the properties of the membrane insertion of the paramyxovirus HN proteins are of interest. There has, however, been no report of the membrane integration of a paramyxovirus HN protein in a cell-free system.

We show here that the HN protein of NDV was inserted into membranes cotranslationally and that insertion required SRP. Significant posttranslational insertion did not occur. In addition, SRP inhibited the translation of HN protein in the absence of membranes. We also found that the HN protein made in the cell-free system was in a somewhat different conformation than was the newly synthesized HN protein in infected cells.

MATERIALS AND METHODS

Cells and viruses. Secondary chicken embryo cells were prepared as described previously (21). Chinese hamster ovary (CHO) cells were grown as described previously (36).

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NDV (Australia-Victoria strain) was propagated and purified as described previously (21).

Preparation of virus-specific RNA. CHO cells were infected with NDV at a multiplicity of infection of 15 PFU per cell. At 6 h postinfection, cells were pelleted, washed once in ice-cold phosphate-buffered saline (0.15 M NaCl, 0.003 M KCl, 0.01 M Na_2HPO_4 , 0.002 M KH_2PO_4) containing 0.01 M adenosine vanadyl complex (3), pelleted, and suspended in RSE (0.01 M Tris hydrochloride [pH 7.4], 0.01 M NaCl, 0.02 M EDTA) containing 1% Triton X-100. RNA was isolated by the methods of Leppert et al. (25) and Dowling et al. (12). Briefly, cytoplasmic extracts were layered over a discontinuous CsCl gradient (3.5 ml of 40% CsCl, 3.5 ml of 20% CsCl, 2 ml of 5% sucrose) and centrifuged in an SW41 rotor at 30,000 rpm for 18 h. The pellet was solubilized in RSE containing 1% sodium dodecyl sulfate and was phenol extracted. The RNA was then ethanol precipitated twice and suspended in water.

Infection and radiolabeling of viral proteins. Monolayers of chicken embryo cells were infected at a multiplicity of 10 PFU per cell. Radiolabeling of infected-cell proteins with [^{35}S]methionine (1,020 Ci/mmol, 14.5 mCi/ml; Amersham Corp., Arlington Heights, Ill.) was accomplished as described previously (28, 29) at 5 h postinfection. Total cytoplasmic extracts were prepared by cell lysis with buffers containing 1% Triton X-100 and 0.5% sodium deoxycholate as described previously (26, 28). For experiments requiring intact cell membranes, infected, radiolabeled cell monolayers were washed with ice-cold phosphate-buffered saline and then with RSB (0.01 M Tris hydrochloride [pH 7.4], 0.01 M NaCl, 0.005 M MgCl_2). Cells were scraped from the plate with a rubber policeman and disrupted with a Dounce homogenizer. Nuclei were removed by a brief centrifugation.

Cell-free protein synthesis. Wheat germ extract was prepared as described by Erickson and Blobel (14).

The reaction mixtures (25 μl) contained 0.75 mM ATP, 140 μM GTP, 13 mM creatine phosphate, 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) (pH 7.8), 1 mM dithiothreitol (DTT), 65 μM amino acids, 60 μg of creatine phosphokinase per ml, 80 μg of spermidine per ml, 56 or 96 mM potassium acetate, and 2.5 mM magnesium acetate. RNase inhibitor (Promega Biotech, Madison, Wis.) was used at 0.5 U/25- μl reaction. Reaction mixtures were incubated for 2 h at 25°C.

Membrane-supplemented translations. Rough microsomal membranes (RM), salt-washed membranes (K-RM), and isolated SRP were prepared as described previously (40). Translation reaction mixtures of 25 μl contained two to three equivalents of membranes per translation or 20 U of SRP per translation. In some cases (see below), protein synthesis was halted by the addition of puromycin (final concentration, 100 μM) after 2 h.

Protease digestion of translation products. Translation products were digested with trypsin at a final concentration ranging from 16 to 333 $\mu\text{g}/\text{ml}$ and with proteinase K at a concentration of 100 $\mu\text{g}/\text{ml}$ (see legend to Fig. 2). Cell extracts were digested with trypsin at a concentration of 16 $\mu\text{g}/\text{ml}$. Digestion was on ice for 1 h, followed by the addition of 32 to 600 μg of soybean trypsin inhibitor per ml to trypsin digestions or phenylmethylsulfonyl fluoride (2 mM) to proteinase K digestions.

Gel filtration of translation products. Translation products were passed over a Sephadex G-50 column equilibrated with 96 mM potassium acetate–2.5 mM magnesium acetate–50 mM triethanolamine hydrochloride [pH 7.4]. Excluded ma-

terial was collected, and aliquots were digested with trypsin as described above.

Polyacrylamide gel electrophoresis. Electrophoresis on sodium dodecyl sulfate-polyacrylamide slab gels was carried out by the procedure of Laemmli (24) with the modifications of Morrison and Ward (29). A 5- μl sample of the translation reaction mixture was applied to each lane. Gels were fixed, dried, and subjected to autoradiography (X-ray film, X-Omat AR; Eastman Kodak Co., Rochester, N.Y.). In some cases (see below), gels were impregnated with PPO (2,5-diphenyloxazole) as previously described (6).

Cleveland peptide analysis. Peptide mapping gels were prepared and electrophoresed by the protocol of Cleveland et al. (8). The radiolabeled proteins were located in a fixed, dried, 10% polyacrylamide gel by autoradiography. The region of the gel containing the protein in question was excised, hydrated in swelling buffer with β -mercaptoethanol, and placed on top of a 12% polyacrylamide gel. The gel slice was overlaid with swelling buffer containing 40% glycerol and then overlaid with swelling buffer containing 12% glycerol and various concentrations of *Staphylococcus aureus* V8 protease (Sigma Chemical Co., St. Louis, Mo.). Electrophoresis was performed as described previously (26).

RESULTS

Translation of NDV RNA in extracts containing membranes. The RNA isolated from NDV-infected cells was used to direct cell-free protein synthesis in wheat germ extracts in the presence or absence of canine pancreas RM. The ^{35}S -labeled reaction products are shown in Fig. 1. Without the addition of microsomal membranes, the expected viral products, i.e., NP (56,000 daltons), P (55,000 daltons), and M (41,000 daltons) proteins, were obtained (9–11), as was a 67,000-dalton polypeptide (lane 3). By tryptic peptide analysis, the 67,000-dalton polypeptide has previously been shown by several laboratories to be unglycosylated HN protein (9–11). When membranes were present during the translation of NDV mRNA, a new 74,000-dalton polypeptide appeared (lane 4) which comigrated with the glycosylated HN protein present in infected-cell lysates (lane 5). This result suggests that HN protein is glycosylated and integrated into the membrane. Translation products from control reactions lacking exogenous mRNA (lanes 1 and 2) did not contain polypeptides with mobilities similar to those of either the unglycosylated or the glycosylated form of the protein.

It is not clear whether F protein was integrated into membranes in these reactions. Glycosylated Fo protein migrates as a 66,000-dalton protein in reducing gels (28). The products of the cell-free reactions containing microsomal membranes did not contain a 66,000-dalton protein product which comigrated with Fo protein from infected cells. The 67,000-dalton polypeptide that was present in these reactions may be either Fo or residual unprocessed HN protein. Further characterization of the translation products with antibody to Fo is required to determine whether Fo protein is being integrated into microsomes in wheat germ extracts.

Protection of the 74,000-dalton protein from digestion. A standard method for determining whether a protein has been inserted into membrane vesicles is to demonstrate its resistance to proteases. The susceptibility of the HN protein to trypsin digestion is shown in Fig. 2. The 67,000-dalton unglycosylated HN protein, as well as NP and P proteins, disappeared when trypsin was added after translation (Fig. 2A, compare lanes 1 and 2). The 74,000-dalton polypeptide

produced in the presence of membranes (Fig. 2B, lane 1) was not reduced in size after trypsin digestion (Fig. 2B, lane 4), yet the two non-membrane-associated proteins NP and P were digested. Identical results were obtained after digestion with proteinase K (Fig. 2B, lane 2). To verify that the protease resistance of the 74,000-dalton protein was due to insertion into the membrane rather than to a protease-resistant conformation in the presence of membranes, we disrupted the membranes with Triton X-100 before digestion. The 74,000-dalton protein completely disappeared when membranes containing the newly synthesized polypeptide were disrupted before proteinase K or trypsin treatment (Fig. 2B, lanes 3 and 5). These results suggest that HN protein can be synthesized and inserted into the microsomal vesicles by using a wheat germ cell-free system. Further support for this conclusion is provided by the increased molecular weight of the presumptive HN in the presence of membranes. This increase is likely due to the addition of core oligosaccharides. The HN protein contains six potential sites for asparagine-linked glycosylation, as deduced from the sequence of the HN gene (McGinnes et al., in press).

Since glycosylated HN protein was completely resistant to protease digestion, there was no evidence that the translocated HN protein was still membrane associated. To verify that the HN protein remained membrane associated after translocation, the reactions were treated with alkali and fractionated on alkaline sucrose gradients as described pre-

FIG. 1. Translation of NDV mRNA in the presence or absence of microsomal membranes. Wheat germ cell-free protein synthesis was carried out at 25°C for 2 h. Aliquots of each reaction were resolved on a 10% sodium dodecyl sulfate-polyacrylamide gel. The dried gel was exposed to X-ray film for 9 h. Lanes: 1 and 2, no added mRNA; 3 and 4, reactions directed by NDV-infected-cell mRNA; 1 and 3, no added RM; 2 and 4, RM present during translation; 5, total ^{35}S -labeled NDV-infected-cell postnuclear supernatant (m). HN_g, HN glycosylated; HN_{ug}, HN unglycosylated.

FIG. 2. HN protein synthesized in the presence of membranes was protected from protease digestion. Wheat germ cell-free synthesis was directed by NDV-infected-cell mRNA in the presence or absence of membranes. Some of the reactions were exposed to trypsin (100 $\mu\text{g}/\text{ml}$) with (A, lane 3; B, lane 5) or without (A, lane 2; B, lane 4) Triton X-100 (0.5%) for 1 h on ice. Trypsin concentrations of 300 and 16 $\mu\text{g}/\text{ml}$ gave identical results. Other reactions were exposed to proteinase K (100 $\mu\text{g}/\text{ml}$) with (B, lane 3) or without (B, lane 2) Triton X-100. Aliquots of each reaction were resolved on polyacrylamide gels as described in the legend to Fig. 1. Memb, Membranes; Pro, protease; Triton, Triton X-100; HN_g, HN glycosylated; HN_{ug}, HN unglycosylated.

viously (2, 18, 30). This protocol causes breakage of vesicles, formation of membrane sheets, and release of soluble components within the vesicle lumen (16). Under these conditions, all translocated HN protein remained membrane associated in a pellet fraction, whereas HN protein synthesized in the absence of membranes remained soluble (data not shown). Thus, the glycosylated HN protein synthesized in vitro was not soluble within the lumen of the microsomal membrane vesicles.

Cleavage peptide analysis of the 74,000-dalton polypeptide. A Cleavage peptide mapping analysis was conducted to verify that the 74,000-dalton polypeptide synthesized in the presence of membranes was glycosylated HN protein. The peptide pattern obtained from the in-vitro-synthesized and membrane-integrated polypeptide was virtually identical to that of authentic glycosylated HN protein derived from infected cells (Fig. 3). Therefore, the inserted 74,000-dalton protein was glycosylated HN protein.

Cotranslational integration of HN into membranes. We next investigated whether HN protein was able to insert into membranes after the cessation of its synthesis. Unglycosylated HN protein was allowed to accumulate during 2 h of cell-free synthesis in the absence of membranes. The translation products were then incubated with membranes for an additional 2 h in the presence of puromycin to assure that any insertion of the unglycosylated HN protein into membranes occurred in the absence of translation. The protein products obtained when NDV-infected-cell RNA was translated in the absence of membranes (Fig. 4, lane 1) were identical to the protein products obtained when membranes were added after protein synthesis was halted (lane 3). No 74,000-dalton HN protein was detected when membranes were added posttranslationally. In addition, the 67,000-

FIG. 3. Cleveand peptide analysis of the 74,000-dalton product synthesized in wheat germ extract. The 74,000-dalton polypeptide bands from fixed, dried gels were digested with 0.25 μ g of V8 protease, and the resulting polypeptide fragments were resolved as described in the text. Lane 1, HN glycosylated protein from infected-cell lysate; lane 2, the 74,000-dalton polypeptide synthesized in the wheat germ translation system programmed with NDV mRNA in the presence of membranes. The slowest-migrating polypeptide is the undigested (74,000-dalton) HN protein.

dalton unglycosylated HN protein remained trypsin sensitive after the posttranslational incubation with membranes in both cases (compare lanes 2 and 4). These results argue that significant amounts of the HN protein are not inserted posttranslationally. In parallel reactions containing membranes during the translation (lanes 6 and 7), the 74,000-dalton HN protein was present and protected from trypsin

FIG. 4. The insertion of HN protein into membranes occurred cotranslationally. Wheat germ protein synthesis was directed by NDV mRNA in the presence or absence of membranes for 2 h. Reactions were stopped by the addition of puromycin to a concentration of 100 μ M. Membranes (Memb) were added where indicated, and extracts were further incubated for 2 h. Aliquots of each sample were digested with trypsin (Tryp) (333 μ g/ml). Lanes: 1 and 2, no membranes; 3 and 4, membranes added posttranslationally (P); 6 and 7, membranes present during the translation (C).

FIG. 5. HN protein required SRP for integration into membranes. Wheat germ cell-free synthesis was directed by NDV RNA in the presence or absence of K-RM, RM, or SRP. Lanes: 1, 3, and 7, no membranes; 2 and 4, K-RM; 6, RM; 3 and 4, 20 U of SRP per 25 μ l; 7, 40 U of SRP per 25 μ l; 5, 35 S-labeled infected-cell lysate as marker (m).

digestion. Therefore, the vast majority of the HN protein was integrated into RM cotranslationally and was not able to insert once the protein was synthesized.

Integration of HN protein into membranes required SRP. The next question to address was the SRP dependence of membrane integration of HN protein. The ability of K-RM devoid of SRP (Fig. 5, lane 2) to integrate and glycosylate HN protein was compared to that of RM (lane 6). K-RM were unable to produce the 74,000-dalton glycosylated HN protein (lane 2). However, the addition of SRP (lane 4) together with K-RM fully reconstituted the glycosylation of HN protein. Therefore, HN protein was unable to integrate into membranes without the cotranslational presence of both membranes and SRP.

Translation of HN protein was inhibited by SRP in the absence of membranes. Many proteins that contain cleavable signal sequences have been shown to undergo translation arrest by SRP in the absence of membranes (42). Not all proteins with uncleaved signal sequences studied to date, however, have demonstrated this same inhibition (2). HN protein synthesis was inhibited in the presence of SRP without membranes (Fig. 5, lane 3) as compared with synthesis in the absence of SRP (lane 1). Scanning densitometry of the autoradiographs of the NDV-specific translation products synthesized in the presence or absence of SRP was used to quantitate the relative amounts of HN and M proteins produced. To compare the results from different translation reactions, the data were normalized by using the M protein as an internal control. The relative amount of unglycosylated HN protein produced in each reaction could then be expressed as the amount (densitometer units) of HN protein per the amount of M protein, which should accurately reflect the influence of SRP on HN protein synthesis (Table 1). The addition of SRP to a translation reaction lacking membranes caused a selective decrease in HN protein synthesis (17 to

27% of the control levels). Similar quantitative values were obtained when NP or P protein was used as the internal control (data not shown). Therefore, the translation of HN mRNA was preferentially inhibited in the presence of SRP without membranes.

Membrane integration of HN protein in NDV-infected cells. The transmembrane nature of the NDV HN protein in infected-cell membranes has not been characterized. Therefore, it is unknown whether the results presented above reflect the situation in infected cells. It has, however, been reported by Schwalbe and Hightower (34) that trypsin digestion of Triton X-100-treated membranes isolated from NDV-infected cells reduces the molecular weight of HN protein from 74,000 to 67,000. They concluded that the HN protein in infected cells has a trypsin-resistant core of 67,000 daltons. In the results presented above, no large trypsin-resistant HN protein fragment was seen. Therefore, the membrane insertion of HN protein in infected cells was explored.

Infected cells were pulse-labeled for 15 min, and total cytoplasmic extracts were prepared as described in Materials and Methods. The extracts, which contain sealed membrane vesicles, were digested with trypsin in the presence or absence of Triton X-100 (Fig. 6, lanes 2 and 3). In contrast to results obtained in the cell-free system, the HN protein was clearly slightly reduced in size by trypsin digestion, suggesting that in infected cells a small region of the HN protein is exposed in a trypsin-sensitive conformation on the cytoplasmic surface of intracellular membranes.

Trypsin digestion of intracellular membranes in the presence of Triton X-100 resulted in a 67,000-dalton fragment (indicated with a dot in Fig. 6, lane 3), as was previously reported (34). That this fragment was indeed derived from the HN protein was verified by immunoprecipitation of the fragment with a monoclonal antibody specific for the HN protein (data not shown). The presence of a 67,000-dalton trypsin-resistant core in infected cells but not in the cell-free system argues that the HN protein is in a different conformation in the two systems. A major difference between the two systems was the presence of a reducing agent in the cell-free reactions. Therefore, DTT was added to infected-cell extracts before trypsin digestion. The reducing agent had no effect on the digestion of intact membranes (Fig. 6, lane 4). However, in the presence of the reducing agent, no 67,000-dalton polypeptide was seen after trypsin digestion of detergent-permeabilized membranes (lane 5). Therefore, the 67,000-dalton trypsin-resistant core found in infected cells

FIG. 6. Conformational differences between the HN protein in infected cells and in the cell-free system. Lanes 1 to 5 contain proteins isolated from infected cells. A monolayer of infected chicken embryo cells (2×10^5 cells) was radiolabeled for 15 min, and cytoplasmic extracts containing intact membranes were prepared by Dounce homogenization as described in Materials and Methods. Lane 1 contains proteins in untreated cell extracts. Extracts were digested with 16 μ g of trypsin per ml (lanes 2 to 5) in the absence (lanes 2 and 4) or presence (lanes 3 and 5) of 1% Triton X-100. DTT (5 mM) was added to extracts in lanes 4 and 5. Lanes 6 and 7 contain products of a cell-free reaction which were subjected to gel filtration. They are, therefore, designated $-^*$ DTT to denote removal of DTT by gel filtration. Membranes were digested with trypsin (16 μ g/ml) in the absence (lane 6) or presence (lane 7) of Triton X-100 (1%).

was likely due to the presence of intramolecular or intermolecular disulfide bonds.

It is possible that the cell-free product also contains inter- or intramolecular disulfide bonds but that the DTT present in the cell-free translation system disrupts these bonds upon membrane permeabilization. To test this possibility, the DTT was removed after synthesis by gel filtration. Membranes were then permeabilized with Triton X-100, and the proteins present were digested with trypsin. Under these conditions, the 67,000-dalton trypsin-resistant core was still not detected (Fig. 6, lane 7).

DISCUSSION

Membrane integration of HN protein. NDV mRNA can direct the translation of the unglycosylated HN protein (67,000 daltons) in wheat germ cell-free synthesis reactions (9–11). We found that the addition of RM to the reaction resulted in the appearance of a new polypeptide with a molecular weight of 74,000 that was identical by Cleveland peptide analysis to glycosylated HN protein derived from infected cells. The 74,000-dalton HN protein was shown to be inserted into microsomal vesicles, since the size of the polypeptide was unchanged after trypsin or proteinase K digestion. When the vesicles were disrupted by detergent before protease digestion, the 74,000-dalton HN protein was no longer detectable.

Our results also showed that translocation of the luminal domain of the HN protein across the microsomal membrane bilayer occurred cotranslationally in the *in vitro* system and required the presence of exogenous SRP. In addition, significant SRP-mediated inhibition of HN protein synthesis occurred in the wheat germ system in the absence of mem-

TABLE 1. SRP-mediated inhibition of HN protein synthesis^a

Expt	SRP ^b	HN/M protein ratio	% of control ^c
1	—	1.55	27
	+	0.42	
2	—	1.62	17
	+	0.28	
3	—	1.50	24
	+	0.36	
4	—	1.49	21
	+	0.31	

^a Reactions similar to those shown in Fig. 5 were subjected to polyacrylamide gel electrophoresis and autoradiography. The amounts of HN and M proteins were quantitated by using a microdensitometer to determine the ratio of the proteins in each reaction.

^b 20 U of SRP per 25- μ l reaction.

^c The HN/M ratio in the absence of SRP is the control or 100%.

branes. This result is similar to those obtained by Spiess and Lodish (35) and Holland and Drickamer (22) for synthesis of the asialoglycoprotein receptor, another transmembrane protein with an amino-terminal anchor. In contrast, the synthesis of the Ca ATPase of sarcoplasmic reticulum and the lens MP26 protein in wheat germ extracts is not inhibited by SRP (2). These proteins also lack a cleaved amino-terminal signal sequence (2). Therefore, in the absence of membranes, SRP may or may not induce a substantial inhibition of synthesis of this class of integral membrane proteins. It is thus far unclear why the synthesis of some proteins in wheat germ extracts is inhibited by SRP and the synthesis of others is not inhibited. Perhaps, the amino acids within or surrounding the signal sequence site play a role in the affinity of SRP binding and therefore influence its effect on protein synthesis.

Membrane assembly of HN protein in infected cells. Trypsin digestion of membranes derived from NDV-infected cells yielded two results different from those obtained in the cell-free system. First, digestion of membranes isolated from infected cells resulted in a slight reduction in the molecular weight of the HN protein, unlike the complete resistance to digestion of the HN protein inserted into microsomal membranes in the cell-free system. The reason for this difference is unclear. The amino acid sequence of the amino-terminal end of the HN protein predicted by analysis of HN cDNA clones contains several trypsin-sensitive residues preceding the putative membrane-spanning region (McGinnes et al., in press). Digestion at these sites should result in a detectable reduction in the molecular weight of the protein, as was found on protease digestion of membranes isolated from infected cells. Thus, the short cytoplasmic tail is in an inaccessible conformation in the cell-free system. In infected cells, the HN protein potentially interacts with other viral proteins or with itself on the cytoplasmic face of the membrane, which may result in a conformation which exposes the cytoplasmic tail to the protease. Alternatively, the HN protein in infected cells may undergo a rapid posttranslational conformational change which makes the cytoplasmic tail more accessible to protease.

A second difference between the cell-free system and infected cells was detected after trypsin digestion of permeabilized membranes. The HN protein made in the cell-free system was completely digested after Triton X-100 treatment of the membranes. In contrast, as has been previously reported (34), the HN protein present in infected cells had a 67,000-dalton trypsin-resistant core in the presence of nonionic detergent. We were able, however, to digest this resistant core after the addition of a reducing agent. This result suggests that the 15-min pulse-labeled HN protein in infected cells contains intramolecular or intermolecular disulfide bonds which protect much of the protein from digestion. It has been previously shown that the HN protein forms intermolecular disulfide bonds resulting in homodimers (34). However, these bonds form 30 to 60 min after synthesis in the *cis* or medial cisternae of the Golgi apparatus (T. Morrison, C. Wilson, and L. McGinnes, manuscript submitted). The membranes used in the present study were prepared from cells radiolabeled for 15 min and contained no detectable labeled HN dimer (data not shown). Therefore, the trypsin-resistant core was most likely due to intramolecular disulfide bonds.

Intramolecular disulfide bond formation occurs in the rough endoplasmic reticulum and is mediated by protein disulfide isomerase (4, 15). It has been reported that these bonds occur cotranslationally or very soon after synthesis

(4, 15). It is possible that the cell-free product does not assume the conformation resulting in a trypsin-resistant core because sufficient reducing agent is trapped within the membrane vesicles during preparation to prohibit proper formation of intramolecular disulfide bonds. It is also possible that the cell-free reactions may not have the necessary components required for this protein modification, as has been suggested by Scheele and Jacoby (31). Alternatively, the HN protein may undergo a conformational change soon after synthesis in infected cells that does not occur in the cell-free system. In any case, our results suggest that the cell-free product is in a different conformation than is the HN protein detected in cells radiolabeled for 15 min.

In summary, the NDV HN glycoprotein was inserted cotranslationally into membranes in a wheat germ cell-free system. Insertion required SRP. Comparisons of the trypsin digestion products of the HN protein produced in the cell-free system with those from infected cells suggests that the HN protein made in the cell-free system is in a conformation different from that of the newly synthesized protein present in infected cells.

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LITERATURE CITED

1. Alkhatib, G., and D. Briedis. 1986. The predicted primary structure of the measles virus hemagglutinin. *Virology* **150**: 479-490.
2. Anderson, D., K. Mostov, and G. Blobel. 1983. Mechanisms of integration of de-novo-synthesized polypeptides into membranes: signal-recognition particle is required for integration into microsomal membranes of calcium ATPase and of lens MP26 but not cytochrome *b₅*. *Proc. Natl. Acad. Sci. USA* **80**:7249-7253.
3. Berger, S., and C. Birkenmeier. 1979. Inhibition of intractable nucleases with ribonucleoside vanadyl complexes: isolation of messenger ribonucleic acid with resting lymphocytes. *Biochemistry* **18**:5143-5149.
4. Bergman, L., and M. Kuehl. 1979. Formation of an intrachain disulfide bond on nascent immunoglobulin light chains. *J. Biol. Chem.* **254**:8869-8876.
5. Blumberg, B., C. Giorgi, L. Roux, R. Raju, P. Dowling, A. Chollet, and D. Kolakofsky. 1985. Sequence determination of the Sendai virus HN gene and its comparison to influenza glycoproteins. *Cell* **41**:269-278.
6. Bonner, W., and R. Laskey. 1979. A film detection method for tritium-labeled proteins and nucleic acids in polyacrylamide gels. *Eur. J. Biochem.* **46**:83-88.
7. Bos, T., A. Davis, and D. Nayak. 1984. NH_2 -terminal hydrophobic region of influenza virus neuraminidase provides the signal function in translocation. *Proc. Natl. Acad. Sci. USA* **81**:2327-2331.
8. Cleveland, D., S. Fischer, M. Firschner, and U. Laemmli. 1977. Peptide mapping by limited proteolysis in sodium dodecyl sulfate and analysis by gel electrophoresis. *J. Biol. Chem.* **252**:1102-1106.
9. Clinkscales, C. W., M. A. Bratt, and T. G. Morrison. 1978. Synthesis of Newcastle disease virus polypeptides in a wheat germ cell-free system. *J. Virol.* **22**:97-101.
10. Collins, P. L., L. E. Hightower, and L. A. Ball. 1978. Transcription and translation of Newcastle disease virus mRNAs in vitro. *J. Virol.* **28**:324-336.
11. Collins, P. L., G. W. Wertz, L. A. Ball, and L. E. Hightower. 1982. Coding assignments of the five smaller mRNAs of Newcastle disease virus. *J. Virol.* **43**:1024-1031.
12. Dowling, P., C. Giorgi, L. Roux, L. Dethlefsen, M. Galantowicz, B. Blumberg, and D. Kolakofsky. 1983. Molecular cloning of the

- 3'-proximal third of Sendai virus genome. *Proc. Natl. Acad. Sci. USA* **80**:5213-5216.
13. Elango, N., J. E. Coligan, R. C. Jambou, and S. Venkatesan. 1986. Human parainfluenza type 3 virus hemagglutinin-neuraminidase glycoprotein: nucleotide sequence of the mRNA and limited amino acid sequence of the purified protein. *J. Virol.* **57**:481-489.
 14. Erickson, A., and G. Blobel. 1983. Cell-free translation of messenger RNA in a wheat germ system. *Methods Enzymol.* **96**:38-50.
 15. Freedman, K. 1984. Native disulfide bond formation in protein biosynthesis: evidence for the role of protein disulfide isomerase. *Trends Biochem. Sci.* **9**:438-441.
 16. Fujiki, Y., A. Hubbard, S. Fowler, and P. Lazarow. 1982. Isolation of intracellular membranes by means of sodium carbonate treatment: application to endoplasmic reticulum. *J. Cell Biol.* **93**:97-102.
 17. Gilmore, R., and G. Blobel. 1983. Transient involvement of signal recognition particle and its receptor in the microsomal membrane prior to protein translocation. *Cell* **35**:677-685.
 18. Gilmore, R., and G. Blobel. 1985. Translocation of secretory proteins across the microsomal membrane occurs through an environment accessible to aqueous perturbants. *Cell* **42**:497-505.
 19. Gilmore, R., P. Walter, and G. Blobel. 1982. Protein translocation across the endoplasmic reticulum. II. Isolation and characterization of the signal recognition particle receptor. *J. Cell Biol.* **95**:470-477.
 20. Hiebert, S. W., R. G. Paterson, and R. A. Lamb. 1985. The hemagglutinin-neuraminidase protein of the paramyxovirus simian virus 5: nucleotide sequence of the mRNA predicts an N-terminal membrane anchor. *J. Virol.* **54**:1-6.
 21. Hightower, L. E., and M. A. Bratt. 1975. Protein metabolism during the steady state of Newcastle disease virus infection. I. Kinetics of amino acid and protein accumulation. *J. Virol.* **15**:696-706.
 22. Holland, E. C., and K. Drickamer. 1985. Signal recognition particle mediates the insertion of a transmembrane protein which has a cytoplasmic NH₂ terminus. *J. Biol. Chem.* **261**:1286-1292.
 23. Kyte, J., and R. Doolittle. 1982. A simple method for displaying the hydrophobic character of protein. *J. Mol. Biol.* **157**:105-132.
 24. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)* **227**:680-685.
 25. Leppert, M., L. Rittenhouse, J. Penault, D. Summers, and D. Kolakofsky. 1979. Plus and minus strand leader RNAs in negative strand virus infected cells. *Cell* **18**:735-747.
 26. McGinnes, L., A. Semerjian, and T. Morrison. 1985. Conformational changes in Newcastle disease virus fusion glycoprotein during intracellular transport. *J. Virol.* **56**:341-348.
 27. Meyer, D., E. Krause, and B. Dobberstein. 1982. Secretory protein translocation across membranes: the role of the "docking protein." *Nature (London)* **297**:647-650.
 28. Morrison, T., L. J. Ward, and A. Semerjian. 1985. Intracellular processing of the Newcastle disease virus fusion glycoprotein. *J. Virol.* **53**:851-857.
 29. Morrison, T., and L. Ward. 1984. Intracellular processing of the vesicular stomatitis virus glycoprotein and the Newcastle disease virus hemagglutinin-neuraminidase glycoprotein. *Virus Res.* **1**:225-239.
 30. Perara, E., and V. Lingappa. 1985. A former amino terminal signal sequence engineered to an internal location directs translocation of both flanking domains. *J. Cell Biol.* **101**:2292-2301.
 31. Scheele, G., and R. Jacoby. 1982. Conformational changes associated with proteolytic processing presecretory protein allow glutathione-catalyzed formation of native disulfide bonds. *J. Biol. Chem.* **257**:12277-12282.
 32. Schneider, C., M. Owen, D. Banville, and J. Williams. 1984. Primary structure of the human transferrin receptor deduced from the mRNA sequence. *Nature (London)* **311**:675-678.
 33. Schuy, W., W. Garten, D. Linder, and H.-D. Klenk. 1984. The carboxyterminus of the hemagglutinin-neuraminidase of Newcastle disease virus is exposed at the surface of the viral envelope. *Virus Res.* **1**:415-426.
 34. Schwalbe, J. C., and L. E. Hightower. 1982. Naturation of the envelope glycoproteins of Newcastle disease virus on cellular membranes. *J. Virol.* **41**:947-957.
 35. Spiess, M., and H. Lodish. 1986. An internal signal sequence: the asialoglycoprotein receptor membrane anchor. *Cell* **44**:177-185.
 36. Stampfer, M., D. Baltimore, and A. Huang. 1969. Ribonucleic acid synthesis of vesicular stomatitis virus. I. Species of ribonucleic acid found in Chinese hamster ovary cells infected with plaque-forming and defective particles. *J. Virol.* **4**:154-161.
 37. Strubin, M., B. Mack, and E. Long. 1984. The complete sequence of the mRNA for the HLA-DR associated invariant chain reveals a polypeptide with an unusual transmembrane polarity. *EMBO J.* **3**:869-872.
 38. Walter, P., and G. Blobel. 1981. Translocation of proteins across the endoplasmic reticulum. II. Signal recognition protein (SRP) mediates the selective binding to microsomal membranes of *in-vitro*-assembled polysomes synthesizing secretory proteins. *J. Cell Biol.* **91**:551-556.
 39. Walter, P., and G. Blobel. 1981. Translocation of proteins across the endoplasmic reticulum. III. Signal recognition protein (SRP) causes signal sequence-dependent and site specific arrest of chain elongation that is released by microsomal membranes. *J. Cell Biol.* **91**:557-561.
 40. Walter, P., I. Ibrahim, and G. Blobel. 1981. Translocation of proteins across the endoplasmic reticulum. I. Signal recognition protein (SRP) binds to *in-vitro*-assembled polysomes synthesizing secretory proteins. *J. Cell Biol.* **91**:545-550.
 41. Wertz, G., P. Collins, Y. Huang, C. Gruber, S. Levine, and L. A. Ball. 1985. Nucleotide sequence of the G protein gene of human respiratory syncytial virus reveals an unusual type of membrane protein. *Proc. Natl. Acad. Sci. USA* **82**:4075-4079.
 42. Wickner, R., and H. Lodish. 1985. Multiple mechanisms of protein insertion into and across membranes. *Science* **230**:400-407.
 43. Yost, C. S., J. Hedgpeth, and V. Lingappa. 1983. A stop transfer sequence confers predictable transmembrane orientation to a previously secreted protein in cell-free systems. *Cell* **34**:759-766.