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Drastic Immunoreactivity Changes between the Immature and Mature Forms of the Sendai Virus HN and F_0 Glycoproteins

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The immunoreactivity of the Sendai virus HN and F_0 glycoproteins was shown to mature before reaching the final form exhibited by the native mature proteins. The maturation process differed for the two proteins. The native F_0 immunoreactivity was shown to be defined cotranslationally, and the addition of high-mannose sugar residues may represent the final step in defining the maturation of immunoreactivity. On the other hand, native HN immunoreactivity was slowly fashioned during the hour after the completion of protein synthesis. Although addition of high-mannose sugar could constitute a necessary step in this slow maturation process, it was shown not to be sufficient. Processing of high-mannose sugars and HN self-association in homodimers and homotetramers were investigated as possible steps involved in the slow maturation of HN immunoreactivity. They were found not to play a significant role. On the other hand, conformational changes presumably took place during the maturation of HN immunoreactivity. Drastic immunoreactivity differences were also demonstrated between the native and denatured forms of the glycoproteins. Possible implications of these results in defining the pathways of glycoprotein synthesis are discussed.

Viral glycoprotein antigens are essential in the immune response to viral infections. They constitute the immunogens against which neutralizing antibodies are raised. Although the pathways by which the glycoproteins are synthesized are reasonably well defined (10, 22), the effect of the modifications that the glycoproteins may undergo during the process of maturation on their final structure and immunoreactivity is poorly understood. For instance, the importance of the carbohydrate residues on the immunoreactivity of the glycoproteins has been investigated only recently (1, 2, 25).

During previous studies of the cell surface expression of the two Sendai virus glycoproteins, HN and F_0, we observed different immunoreactivity behavior for the two proteins during pulse-chase experiments. After a pulse of [^{35}S]methionine, F_0 was readily precipitated by a rabbit heteroantiserum or by monoclonal antibodies, whereas HN was only efficiently recovered by immunoprecipitation after a lag of 30 to 60 min (19, 20), suggesting that the immunoreactivity of the HN protein needed some maturation step.

The present work confirmed the difference in immunoreactivity maturation of HN and F_0 and represented an attempt to characterize the factors involved in the modeling of the native mature immunoreactivity of the two proteins, i.e., the immunoreactivity that the proteins exhibit on the envelope of the virus particles. The evolution of the immunoreactivity of the two viral glycoproteins was measured during [^{35}S]methionine pulse-chase labeling experiments by using preparations of polyclonal and monoclonal antibodies raised against the native mature forms of HN and F_0. The extent of the immunoreactivity of the proteins recovered at different times after the end of the radioactive pulse served as an indication of the correlations between the mature native forms and the immature forms of HN and F_0, i.e., the forms they exhibited soon after the [^{35}S]methionine pulse or during the chase period. These estimations were made under normal conditions and under conditions in which the glycoprotein maturation was perturbed, namely in the presence of tunicamycin, in a mutant cell line exhibiting a defect in the glycosylation pathway, or after deglycosylation of the proteins by endoglycosidase. Self-association of HN in homodimers and homotetramers was also monitored for a putative role in the slow maturation of HN immunoreactivity. In similar experiments, the immunoreactivity of the native proteins was compared with that of their denatured counterparts. These experiments allowed us to conclude that HN and F_0 native mature immunoreactivities are modeled differently. The experiments surprisingly ruled out glycosylation processing and self-association as preponderant factors in modeling the immunoreactivity of HN. In the end, they argue for different metabolic pathways in the maturation of HN and F_0.

MATERIALS AND METHODS

Virus and cells. Sendai virus Harris strain was grown in 9-day-old chicken embryos infected with 1/10 the yield of a virus plaque. After 3 days of incubation at 33°C, the allantoic fluid was collected, clarified (3,000 × *g* for 1 h), and frozen as aliquots at −70°C. BHK-21 cells from the American Type Culture Collection, Rockville, Md., were routinely used. Ric^R 14 cells were obtained from P. Vischer and R. C. Hughes, National Institute for Medical Research, Mill Hill, London, England; they are ricin-resistant BHK cells with very low levels of *N*-acetylglucosamine transferase I (30). The cell lines were routinely grown in Eagle minimal essential medium supplemented with 5% heat-inactivated fetal bovine serum under a 5% CO_2 atmosphere.

Sendai virus rabbit antisera and monoclonal antibodies. Rabbit serum against the whole purified Sendai virus (RAb-vir) was prepared by a series of subcutaneous and intramuscular injections of UV-inactivated egg-grown virus emulsified in complete Freund adjuvant. Rabbit sera raised against sodium dodecyl sulfate (SDS)-denatured HN and F_0 proteins (RAb-HN_{SDS} and RAb-$F_{0_{SDS}}$) were obtained by injecting the rabbits with the protein purified by polyacrylamide gel electrophoresis. The protein was localized on the gel by

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Coomassie blue staining, excised, mashed, and injected subcutaneously as a gel homogenate. Monoclonal antibodies derived from mouse hybridoma cell lines secreting anti-HN or anti-F_0 antibodies were prepared as described previously (17). The anti-HN monoclonal antibodies S-16, M-11, M-9, and M-21 have been shown to specifically precipitate HN and to react with the protein at the surface of infected cells (19). The anti-F_0 monoclonal antibodies M-16 and M-33 have been shown to specifically precipitate F_0, but only M-16 has been shown to react with the protein at the surface of the cell (19). M-38 was shown in the present study to react with F_0. The further characterization of all of these monoclonal antibodies and the demonstration that they are directed against nonoverlapping epitopes, as determined by competitive binding assays, will be presented in separate papers (A. Portner, manuscripts in preparation).

Pulse-chase labeling of infected cells with [^{35}S]methionine. Infected cells were incubated for 15 min with methionine-free minimal essential medium and then labeled for 15 min with 300 μCi of [^{35}S]methionine (700 to 1,000 Ci/mmol; Amersham Corp., Arlington Heights, Ill.) per ml. At the end of the labeling period, the cells were washed twice with regular minimal essential medium containing 10 mM cold methionine and 2% fetal bovine serum and either harvested after two successive washes with cold phosphate-buffered saline (PBS) or incubated in cold methionine-enriched medium for various periods of time before harvest (see Results).

Immunoprecipitations. Cell surface immunoprecipitations were performed as follows (21). [^{35}S]methionine-pulse-labeled and chased infected cells were washed with cold PBS when still attached to the growth surface and were then allowed to react with the antibody preparations (diluted in PBS) for 2 h at 4°C. The cells were then washed twice with cold PBS to remove excess antibody, disrupted in Triton-SDS buffer (150 mM NaCl, 1% deoxycholate, 1% Triton X-100, 0.1% SDS, 10 mM Tris hydrochloride [pH 7.8]), sonicated at 40 W for 15 s (Branson Sonic Power Co., Danbury, Conn.), and centrifuged for 15 min in an Eppendorf centrifuge. The supernatants (cellular extracts) were collected and incubated for 1 h at 4°C with protein A-Sepharose CL-4B (Pharmacia, Inc.). The Sepharose beads were then washed twice with 150 mM NaCl–5 mM EDTA–50 mM Tris hydrochloride [pH 7.8]–0.5% Nonidet P-40 and once with 0.5 M LiCl–0.1 M Tris hydrochloride [pH 7.8]–1% 2-mercaptoethanol and suspended in Laemmli polyacrylamide gel electrophoresis (PAGE; 12) sample buffer. Total cell immunoprecipitations differed from cell surface immunoprecipitations only in that the cellular extracts were prepared before the incubation with the antibody preparations. For total cell immunoprecipitations performed after SDS denaturation and reduction of the proteins, the infected cells were disrupted in 2% SDS–1% 2-β-mercaptoethanol, sonicated, boiled for 3 min, and then diluted 20-fold with the Triton-SDS buffer before incubation with the antibodies. The immunoprecipitated proteins were analyzed by PAGE (12).

Endo-H digestion. Immunoprecipitated viral proteins were suspended in 1% SDS–50 mM Tris hydrochloride [pH 6.8]–0.5% 2-β-mercaptoethanol–2 mM phenylmethylsulfonyl fluoride and boiled for 5 min. The protein solutions were then diluted 10-fold with 125 mM sodium citrate (pH 5.0) containing 2 mM phenylmethylsulfonyl fluoride, and identical samples were either digested overnight at 37°C with 80 mU of endo-β-*N*-acetylglucosaminidase-H (endo-H) (Nenzymes) per ml or mock treated. The proteins were then concentrated by acetone precipitation (8:1, vol/vol) and analyzed by PAGE.

Endo-F and trypsin digestions. [^{35}S]methionine-pulse-labeled and chased infected cells were disrupted in the total cell immunoprecipitation buffer at pH 7.8, and the cytoplasmic extracts were prepared as described for total cell immunoprecipitation. Samples, each containing about 2.5×10^6 cells, were treated with 20 μl of an endo-F preparation (7) (a gift from J. Kaufmann, Immunology Institute, Basel, Switzerland) for 30 min at room temperature. After the digestion, the proteins were recovered by immunoprecipitations (see Results). For trypsin digestions, samples of cytoplasmic extracts, each containing 10^6 cells, were digested at room temperature with 0.7 μg of N-acetylated trypsin (Sigma Chemical Co., St. Louis, Mo.) for increasing periods of time. The digestion was stopped by the addition of 5 mM phenylmethylsulfonyl fluoride and 1% aprotinin. The samples were made 2% in SDS and 1% in β-mercaptoethanol, boiled for 3 min, diluted 20-fold with total cell immunoprecipitation buffer, and finally immunoprecipitated with RAb-HN_{SDS} (see above).

Quantitation of viral proteins. Antisera or monoclonal antibodies were titrated to ensure their slight excess in the immunoprecipitations, as previously described (21). Gel autoradiograms of different exposure times were scanned to ensure direct proportionality between the darkening of the film and the amounts of the [^{35}S]methionine-labeled viral proteins. The areas under the scanned peaks were then integrated.

RESULTS

Evolution of the immunoreactivities of the native HN and F_0. First the extent of the immunoreactivity of HN and F_0 was measured as a function of time elapsed after their synthesis. BHK-infected cells were pulse-labeled for 15 min with [^{35}S]methionine and then chased in an excess of nonradioactive methionine for increasing periods of time before harvest (0, 1, or 2 h) and analysis. The immunoreactivity of the glycoproteins was then defined at each time point by immunoprecipitations with different sets of antibodies. For the HN protein, four monoclonal antibodies directed against different epitopes present on the mature native protein were used. HN immunoreactivity evolved with time (Fig. 1A and D); the amount of HN recovered with the four monoclonal antibodies was 5- to 10-fold higher after 1 h of chase than at the end of the pulse. Although all the monoclonal antibodies precipitated more HN after 1 h of chase, they showed diverging abilities to precipitate the protein after a chase period of 2 h; M-9 and in particular M-21 seemed to have lost part of this ability. In contrast to HN, F_0, which was immunoprecipitated with three different monoclonal antibodies, was recovered in similar amounts at all times (Fig. 1B and E). Similarly, all of the other viral proteins immunoprecipitated with RAb-vir behaved like F_0, except HN, whose increased recovery after 1 h of chase was also seen when the rabbit antiserum was used (Fig. 1C and F), although the difference between the yield at 0 and 1 h was smaller than that observed with the monoclonal antibodies.

Immunoreactivity of the native and denatured HN and F_0. In the experiments presented above (Fig. 1), the immunoreactivity of only the native proteins was followed as a function of time. In that way, the immunoreactivity of HN was shown to change significantly. To try to estimate the extent of this change or to detect other possible modulations, the immunoreactivity of the native viral proteins was com-

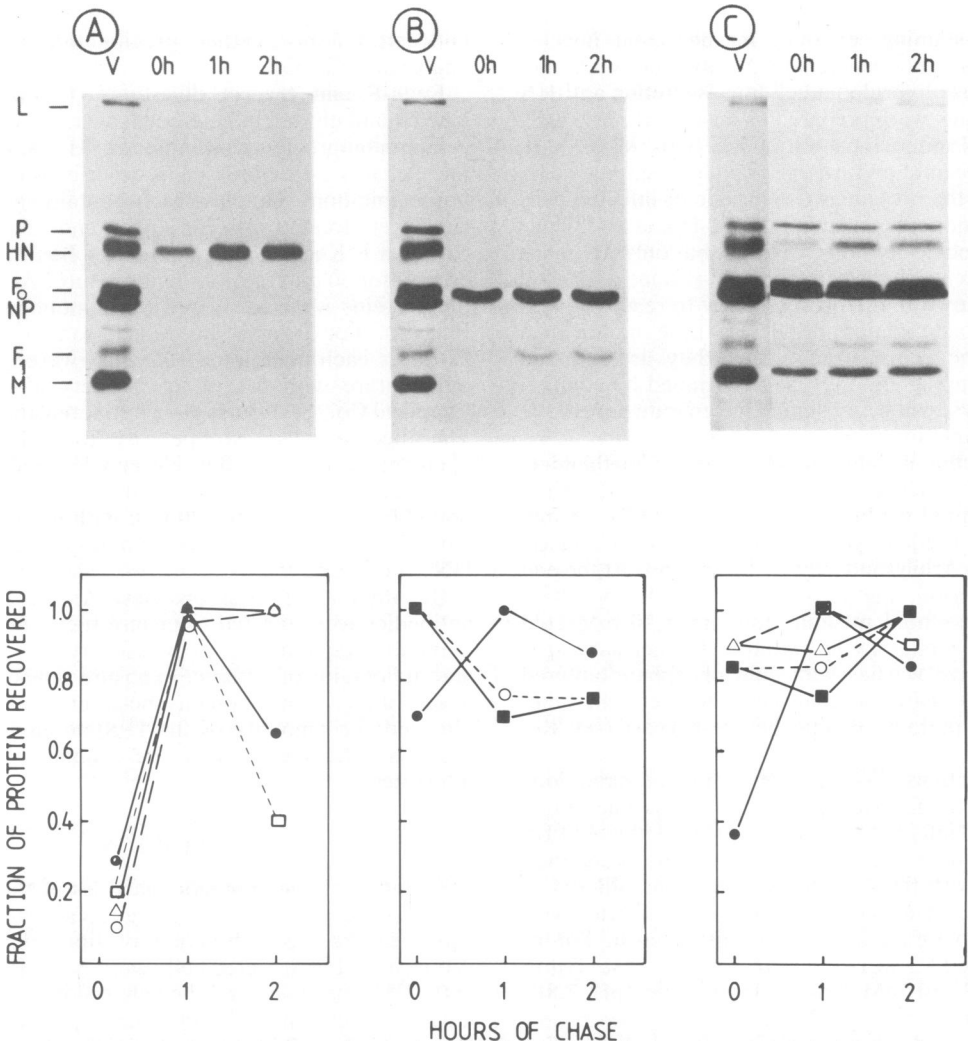

FIG. 1. Time course analysis of immunoreactivity of the viral proteins synthesized in BHK cells. Infected BHK cell samples (10^7 cells) were pulse-labeled for 15 min with [^{35}S]methionine and chased for 0, 1, and 2 h. Total cell immunoprecipitations were performed with 1/10 the cellular extract of a cell sample, 3 μl of each of the monoclonal antibodies, and 5 μl of RAb-vir in 200-μl reaction mixtures. A 50% suspension of protein A-Sepharose (60 μl) was then used to recover the immunoprecipitates, which were then analyzed by 10% PAGE. Autoradiograms: PAGE of the immunoprecipitates obtained with anti-HN (S-16) (A) and anti-F_0 (M-38) (B) monoclonal antibodies and with RAb-vir (C). (D to F) Computation of the viral protein amounts precipitated by anti-HN monoclonal antibodies (D) (○, S-16; △, M-11; ●, M-9; □, M-21), anti-F_0 monoclonal antibodies (E) (●, M-16; ○, M-38; ■, M-33), and RAb-vir (F) (●, HN; ○, polymerase-associated protein [P]; ■, F_0; △, nucleoprotein [NP]; □, matrix protein [M]). Fraction 1.0 was defined as the maximum yield of the protein. V, Viral protein markers; L, large polymerase.

pared with that of their denatured and reduced counterparts. The analysis of the immunoprecipitates obtained after SDS denaturation and 2-β-mercaptoethanol reduction of the cytoplasmic extracts is presented in Fig. 2. There was a noticeable contrast between the efficient reactivity of the heteroclonal RAb-vir and the monoclonal antibodies (S-16 [anti-HN] and M-33 [anti-F_0]) with the native HN and F_0 proteins (Fig. 2A and B, −SDS lanes), and the absence of reactivity of their denatured counterparts with the same antibodies (Fig. 2A and B, +SDS lanes). It is noteworthy that the absence of reactivity of the denatured HN with either the heteroclonal or monoclonal antibodies was observed for HN recovered just after the pulse of [^{35}S] methionine (lanes 0), as well as 1 h later (lanes 1), and therefore did not depend on HN immunoreactivity evolution (as shown in Fig. 1). The reactivity of the RAb-HN_{SDS} and RAb-$F_{0_{SDS}}$ was tested against the denatured and nondenatured proteins. These antibodies reacted with the native (−SDS lanes) as well as with the denatured (+SDS lanes) glycoproteins (Fig. 2C). The increase in reactivity of HN observed previously during the first hour after the radioactive pulse did not occur with the use of the RAb-HN_{SDS}. This presumably reflected the fact that the denaturation of the protein abolished the differences in immunoreactivity between the native protein analyzed just after the pulse of [^{35}S]methionine and that analyzed 1 or 2 h later (Fig. 1). This also ruled out the possibility that the poor recovery of HN in immunoprecipitations done at time zero of chase (Fig. 1) resulted from an inefficient labeling of the protein (due to an unusually slow rate of synthesis) and confirmed that the further increase in the yield of the immunoprecipitations did not result from further incorporation of the radioactive precursor in HN during the chase but from a maturation of antigenic sites, creating efficient antibody-protein recogni-

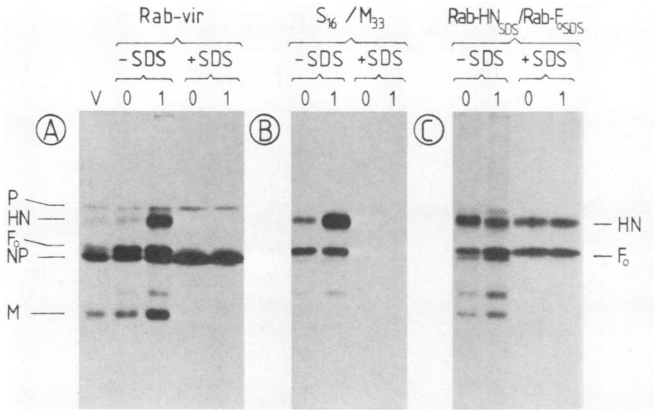

FIG. 2. Comparison of immunoreactivity of HN and F_0 in denatured or nondenatured forms. At 18 h postinfection, infected BHK cell samples (10^7 cells) were pulse-labeled with [^{35}S]methionine for 15 min and further chased for 0 and 1 h. Samples, each containing 10^6 cells, were used directly in total cell immunoprecipitations (−SDS samples); similar samples were boiled for 3 min in PAGE sample buffer (12) and then diluted 20 times to allow reaction with the antibodies (+SDS samples). In both types of reaction, identical concentrations of antibodies were used, a 1:10 dilution for RAb-vir (A), a 1:40 dilution for the mixture of monoclonal antibodies (S-16 and M-33) (B), and a 1:10 dilution for the mixture of RAb-HN_{SDS} and RAb-F_{0SDS}. A 50% suspension of protein A-Sepharose (60 μl) was then used to recover the immunoprecipitates, which were analyzed by 10% PAGE. Abbreviations are defined in the legend to Fig. 1.

tion. Direct analysis of the cytoplasmic extracts by PAGE confirmed this conclusion by showing no variations in the amount of HN relative to the amounts of the other viral proteins during the chase periods (Fig. 3).

Immunoreactivity of nonglycosylated HN and F_0. HN, as well as F_0, are viral glycoproteins expressed at the surface of infected cells where the viral particles are budding (6) and exclusively contain Asn-N-linked glycans (11, 31). To define the contribution of the sugar residues to the formation of the immunoreactivity of the native mature proteins, immunoreactivity was studied when the proteins were synthesized in the presence of tunicamycin. Tunicamycin blocks the synthesis of the high-mannose glycan on the dolichol lipid which donates the sugar to the proteins, so that the proteins synthesized in the presence of the drug are not glycosylated (28). PAGE of the proteins immunoprecipitated from infected BHK cells grown and pulse-labeled in the presence of tunicamycin showed that the two usual glycoproteins were missing and were replaced by two new proteins which migrated faster than did HN and F_0 (Fig. 4A). These two proteins, HN_{TM} and $F_{0_{TM}}$, have been shown to be the unglycosylated forms of HN and F_0 (15). At 0 h the two unglycosylated proteins were immunoprecipitated with an efficiency equivalent to that of the immunoprecipitation of the glycosylated proteins with the RAb-vir. Surprisingly, after 2 h of chase, almost no HN and much less F_0 were immunoprecipitated with the same serum (Fig. 4A). This contrasted with the expected recovery observed after 2 h of chase in the control cells (Fig. 4A). Moreover, the unglycosylated proteins had completely lost the ability to be precipitated by the anti-HN and anti-F_0 monoclonal antibodies regardless of the time of analysis (Fig. 4B and C); these results were verified for the four anti-HN and three anti-F_0 monoclonal antibodies (data not shown). In a complementary experiment (data not shown), the use of the RAb-HN_{SDS} and RAb-$F_{0_{SDS}}$ showed that the recovery of HN_{TM} and $F_{0_{TM}}$ similarly decreased with increasing time of chase. Moreover, control experiments were done to exclude the possibility that HN_{TM} and $F_{0_{TM}}$ aggregated and were lost during the preparation of the cytoplasmic extracts. Therefore, HN_{TM} and $F_{0_{TM}}$ were presumably degraded faster after their synthesis.

Role of glycosylation processing in maturation of native HN and F_0 immunoreactivities. The experiments presented above demonstrated that the immunoreactivity of the native HN and F_0 proteins differed drastically from that of their mature denatured counterparts and from that of the proteins made in the presence of tunicamycin. The data indicated that the native immunoreactivity could be fashioned after some maturation step occurring at or after the addition of the high-mannose sugar residues. It then became important to estimate the role that the glycosylation and its processing may play on the definition of the native glycoprotein immunoreactivities. Therefore, the sensitivity of HN and F_0 to endo-H was estimated, because sensitivity or resistance to endo-H reflects maturation of the sugar residues from high-mannose glycans to complex sugars (29). HN recovered by

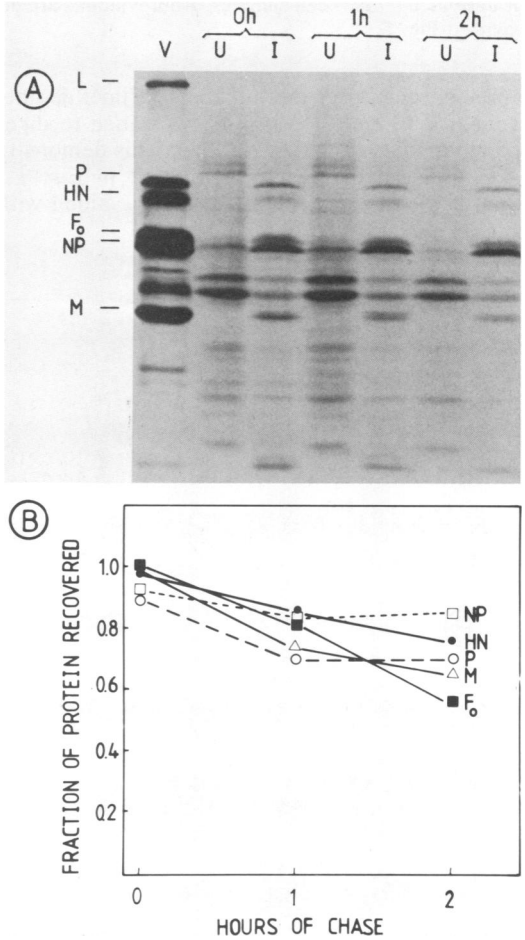

FIG. 3. Uninfected (U) and Sendai virus-infected (I) BHK cells were pulse-labeled and chased for 0, 1, and 2 h as described in the legend to Fig. 1. Cellular extracts were prepared as described in Materials and Methods, and samples were directly dissolved in Laemmli PAGE buffer and analyzed by PAGE. (A) Autoradiogram of PAGE. (B) Computation of the amount of viral proteins (mean of two experiments). Fraction 1.0 was taken as the highest amount of estimated protein. Abbreviations are defined in the legend to Fig. 1.

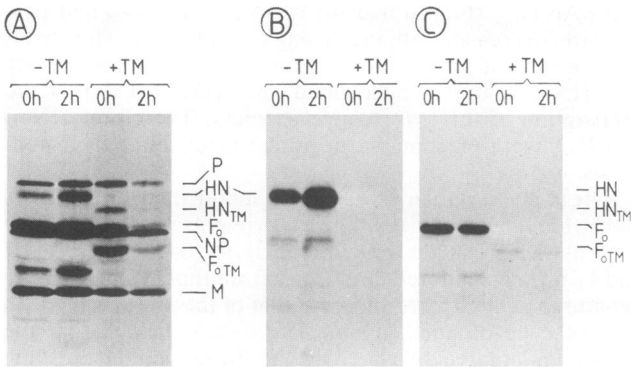

FIG. 4. Effect of tunicamycin on HN and F_0 immunoreactivity. Infected BHK cell samples were pulse-labeled with [^{35}S]methionine and chased for 0 and 2 h, as described in the legend to Fig. 1, except that, for half of the samples, 0.25 μg of tunicamycin per ml was constantly present. Total cell immunoprecipitations were performed as described in the legend to Fig. 1, with S-16, M-16, or RAb-vir, and the immunoprecipitates were analyzed by PAGE. Autoradiograms of immunoprecipitates obtained with RAb-vir (A), S-16 (anti-HN) (B), and M-16 (anti-F_0) (C) from tunicamycin-treated (+TM) or untreated (−TM) cell samples. Abbreviations are defined in the legend to Fig. 1.

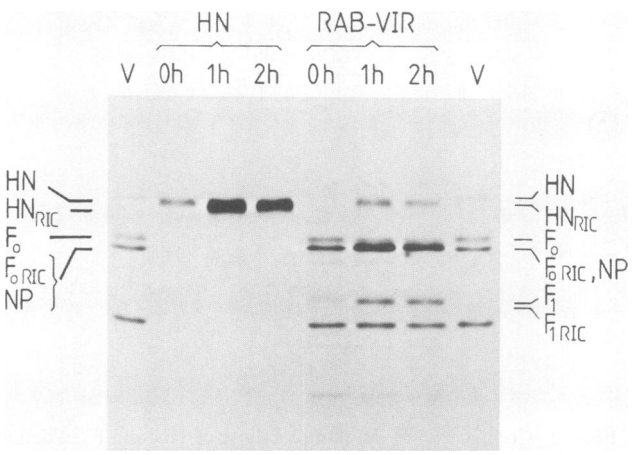

FIG. 6. Time course analysis of immunoreactivity of viral proteins synthesized in Ric^R 14 cells. The procedures were the same as those described in the legend to Fig. 1, except that Ric^R 14 cells were used in place of normal BHK cells. M-11 (anti-HN) and RAb-vir were used. HN_{RIC} and F_{0RIC}, mature HN and F_0 made in Ric^R cells. Abbreviations are defined in the legend to Fig. 1.

immunoprecipitation after the pulse of [^{35}S]methionine was totally sensitive to endo-H, and its resistance to digestion increased gradually with time (Fig. 5A). This demonstrated, in conjunction with the results presented in Fig. 1, that maturation of the sugar residues took place along with the maturation of the native immunoreactivity. However, the results presented in Fig. 5A, in conjunction with those presented in Fig. 1, also demonstrated that HN could be, at least partly, immunoprecipitated when it contained only high-mannose glycans and that it was not necessarily completely resistant to endo-H when it was efficiently immunoprecipitated (after 1 h of chase). F_0 resistance to endo-H somehow evolved faster (Fig. 5B), and this experiment, in conjunction with results presented in Fig. 1, demonstrated that F_0 native immunoreactivity was fully determined on the protein after the addition of the high-mannose glycans and was not affected by further sugar processing.

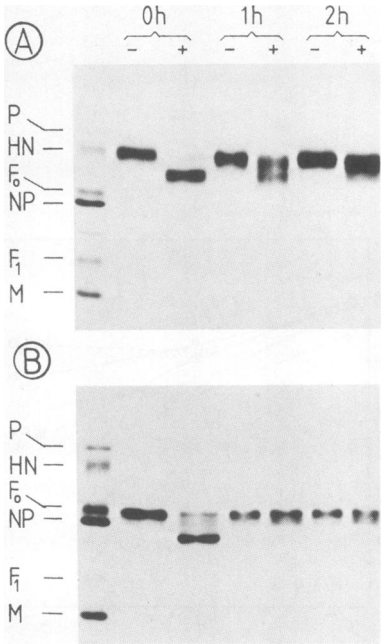

FIG. 5. Endo-H digestion of [^{35}S]methionine-pulse-labeled and chased HN and F_0. HN and F_0 were immunoprecipitated from infected BHK cell samples that had been pulse-labeled with [^{35}S]methionine and chased for 0, 1, and 2 h, as described in the legend to Fig. 1. Samples of the proteins were digested with endo-H (+) (see Materials and Methods) and analyzed by PAGE along with mock-digested samples (−). (A) HN samples (precipitated with S-16); (B) F_0 samples (precipitated with M-16). Abbreviations are defined in the legend to Fig. 1.

In an attempt to more precisely define a possible effect for sugar residue processing on the maturation of native HN immunoreactivity, maturation was followed in Ric^R 14 cells. These cells lack *N*-acetylglucosamine transferase I (30); as a result, glycosylation is blocked during the trimming of the high-mannose sugar Glc_3-Man_9-$GlcNAc_2$ at the Man_5-$GlcNAc_2$ step, so that no complex sugars are ever synthesized. The results of the immunoprecipitations performed in [^{35}S]methionine pulse-chase experiments with these Ric^R 14 cells are presented in Fig. 6. First, the block was effective, because the proteins recovered at 1 and 2 h of chase migrated faster than those recovered at time zero of chase (for example, F_0 made in Ric^R 14 cells [$F_{0_{RIC}}$] migrating at the nucleoprotein [NP] position). Moreover, HN and F_0 never acquired resistance to endo-H in these mutant cells (results not shown). Despite the block in glycosylation, the HN immunoreactivity matured, as indicated by the increase in HN recovery from time zero to 1 or 2 h of chase. This increase was verified with the four anti-HN monoclonal antibodies (results for three not shown), as well as by RAb-vir. This experiment ruled out the possible need for a glycosylation process extending past the block of Ric^R 14 cells in the HN native immunoreactivity maturation. However, it did not exclude the possibility that this maturation might depend on a step situated between high mannose addition and Man_5-$GlcNAc_2$.

To determine whether the low immunoreactivity of HN soon after synthesis was due to steric hindrance or a block in the maturation of epitopes by some sugar residues, HN was

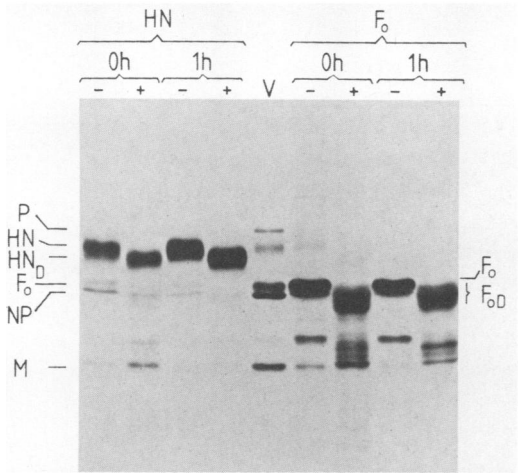

FIG. 7. Digestion of viral glycoproteins with endo-F. Infected BHK cell samples were pulse-labeled with [^{35}S]methionine and chased for 0 and 1 h, as described in the legend to Fig. 1. Cytoplasmic extracts were prepared in 400 μl as for total cell immunoprecipitations, and cell samples were either digested with endo-F (+ samples) or mock treated (− samples) as described in Materials and Methods. Total cell immunoprecipitations were then run with anti-HN (S-16) or anti-F_0 (M-38) monoclonal antibodies (1:12 dilution). The immunoprecipitates were analyzed by 10% PAGE. Abbreviations are defined in the legend to Fig. 1.

deglycosylated by digestion with endo-F immediately after synthesis and before reaction with the antibodies. Despite an effective digestion of HN with endo-F, as evidenced by the change in the apparent molecular weight of the digested protein (Fig. 7), its immunoreactivity analyzed at the end of the radioactive pulse (0 h) was not modified compared with that of its nondigested counterpart (compare 0-h + and − samples). Also relevant was the finding that the immunoreactivity of the protein recovered after 1 h of chase was similarly not altered by sugar residue removal (1-h + and − samples). Therefore, deglycosylation of HN did not alter its immunoreactivity. Endo-F also digested F_0 and generated a heterogeneous population of proteins (Fig. 7), with an amount of immunoprecipitated material corresponding to that obtained with the nondigested protein. It is noteworthy that, although the F_0 native immunoreactivity appeared to be abruptly and completely defined after the adjunction of the high-mannose sugar residues, the immunoreactivity was nevertheless not changed by the subsequent removal of these sugars.

Polymerization of the HN protein as a possible cause of maturation of its antigenic sites. The HN protein is present on the envelope of the virus particle not as a monomer but in the form of disulfide-linked homodimers and homotetramers (13, 27). It is not known when this self-association takes place, and therefore it was not unlikely that if it took place soon after HN synthesis, it could be responsible for the immunoreactivity exhibited by the native mature protein. This possibility was investigated by monitoring the time course of dimer and tetramer formation in parallel with the maturation of the immunoreactivity. Pulse-labeled HN protein was immunoprecipitated after increasing periods of chase and analyzed by PAGE under reducing and nonreducing conditions (Fig. 8). The maturation of the immunoreactivity, expressed as the efficiency of HN recovery in immunoprecipitation (Fig. 9A), and the formation of dimers and tetramers (Fig. 9B) are plotted as a function of the length of the chase. Three of the monoclonal antibodies differed to some degree in the increase in dimer and tetramer formation compared with the increase in recovery of HN (Fig. 9A and B). In the case of M-11, the best example, about 80% of the protein was found in a self-associated form after 60 min of chase. In the case of S-16 and M-9, however, the increase in oligomer formation was not really significant during the maturation of the immunoreactivity, and about 50% of the protein was still a monomer when efficiently immunoprecipitated. In these cases, the kinetics and the extent of polymer formation did not convincingly parallel the immunoreactivity maturation. More important were the results with M-21, for which the immunoreactivity matured without any increase in oligomer formation above the level seen at 0 min of chase (20%). It is noteworthy that, of the four monoclonal antibodies, M-9 and particularly M-21 showed reduced affinity or a lack of affinity for the associated form of HN. This could explain the apparent reversion of reactivity that these antibodies had for HN between 1 and 2 h of chase (Fig. 1), because the maturation of the immunoreactivity for M-9 and M-21 could have been counterbalanced by the formation of increasing amounts of polymerized HN.

Further evidence for conformational changes taking place during HN immunoreactivity maturation. As indicated above, the maturation of HN immunoreactivity could not be satisfactorily explained by structural modifications of the protein such as glycosylation or self-association. In an attempt to demonstrate without the aid of antibodies that, during maturation, HN conformation was changing, sensitivity of HN to digestion by trypsin was investigated. Cytoplasmic extracts prepared from infected cells pulse-labeled and chased for 0 or 1 h were digested with trypsin for increasing periods of time. Immunoprecipitations were then performed with RAb-HN_{SDS} after denaturation of the extracts in SDS, and the immunoprecipitates were analyzed by PAGE to estimate the extent of the trypsin digestion (Fig. 10). Two facts were significant. First, the sensitivity of HN to trypsin decreased with increased time of chase; for example, a 30-min incubation with the enzyme resulted in the digestion of about 90% of HN just after the pulse (Fig. 10; 0 h, 30 min) but only 50% after 1 h of chase (Fig. 10; 1 h, 30 min). Second, the products of the digestion differed; digestion of HN at 0 h generated polypeptides of low molecular weight (20,000 to 30,000), whereas digestion at 1 h almost exclusively yielded a protein slightly smaller than the NP.

DISCUSSION

HN and F_0 are membrane-anchored glycoproteins containing Asn-N-linked sugars (11, 31) and are likely to be synthesized and mature by the general scheme established for such proteins (for a review, see references 10 and 22). They are synthesized by ribosomes attached to the endoplasmic reticulum (ER), with the nascent protein traversing the ER membrane to reach the lumen, where the high-mannose sugars are cotranslationally added. After synthesis is completed, the proteins, still anchored in the ER membrane by hydrophobic domains, travel from the ER through the Golgi apparatus to the plasma membrane, where they are expressed at the cell surface. During this transport, the high-mannose sugars are processed stepwise by the removal of glucose and mannose residues and by subsequent addition of terminal sugars. HN, but not F_0, is found self-associated in disulfide-linked homodimers and homotetramers at the cell surface.

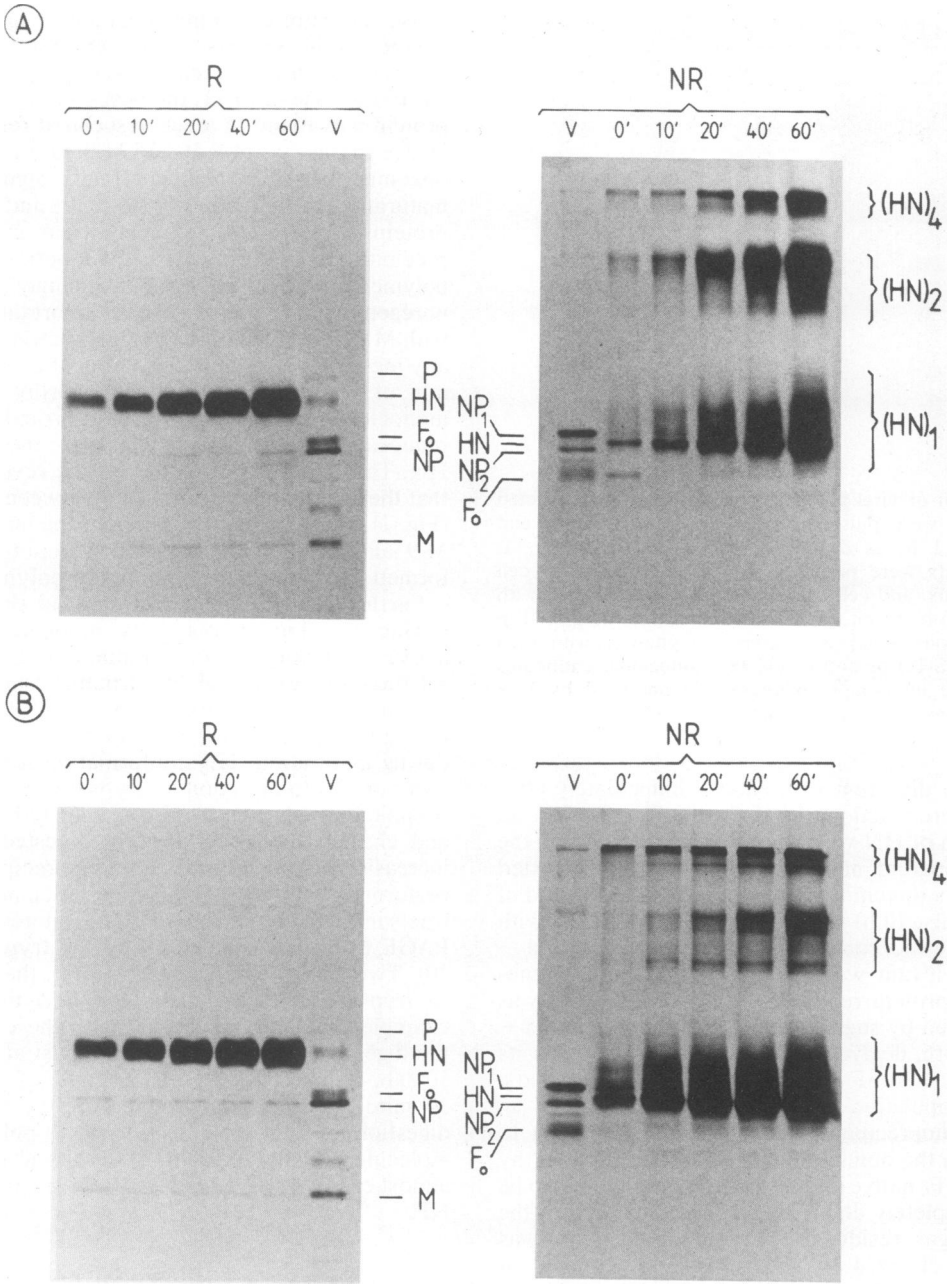

FIG. 8. PAGE, under reducing or nonreducing conditions, of immunoprecipitated HN recovered in pulse-chase experiments. Infected BHK cell samples were pulse-labeled with [^{35}S]methionine as described in the legend to Fig. 1 and chased for 0, 10, 20, 40, or 60 min. HN was immunoprecipitated as described in the legend to Fig. 1 and analyzed by PAGE either after boiling for 3 min in regular PAGE sample buffer (reducing conditions, R samples) or after boiling in sample buffer lacking β-mercaptoethanol (nonreducing conditions, NR samples). Immunoprecipitates were obtained with S-16 (A) and M-21 (B). $(HN)_2$ and $(HN)_4$, HN as a dimer and tetramer, respectively. The identification of the viral proteins under nonreducing conditions was achieved by immunoprecipitation of each protein with specific antibodies and by comparison of their migration patterns under reduced and nonreduced conditions. NP was shown in this way to separate into two distinct forms (NP_1 and NP_2) under nonreduced conditions. Abbreviations are defined in the legend to Fig. 1.

From this scheme it was expected that the addition of high-mannose sugars might participate in the definition of the mature immunoreactivity because sugars represent an important contribution to the final protein mass (9 and 15% of HN and F_0, respectively [11]). The experiments done with tunicamycin indicated that this may indeed be the case for F_0, at least as far as the immunoreactivity defined by the monoclonal antibodies is concerned, because F_0 native immunoreactivity was found fully defined only after the addition of the high-mannose sugars. For HN, on the other hand, this step is not sufficient because HN immunoreactivity still matured after the addition of high-mannose sugars, with a half time of 20 to 30 min. The participation of the carbohydrate chains in defining the immunoreactivity of glycoproteins seems to vary. Basak and Compans (2) reported that "immunoprecipitation reactions involving five different

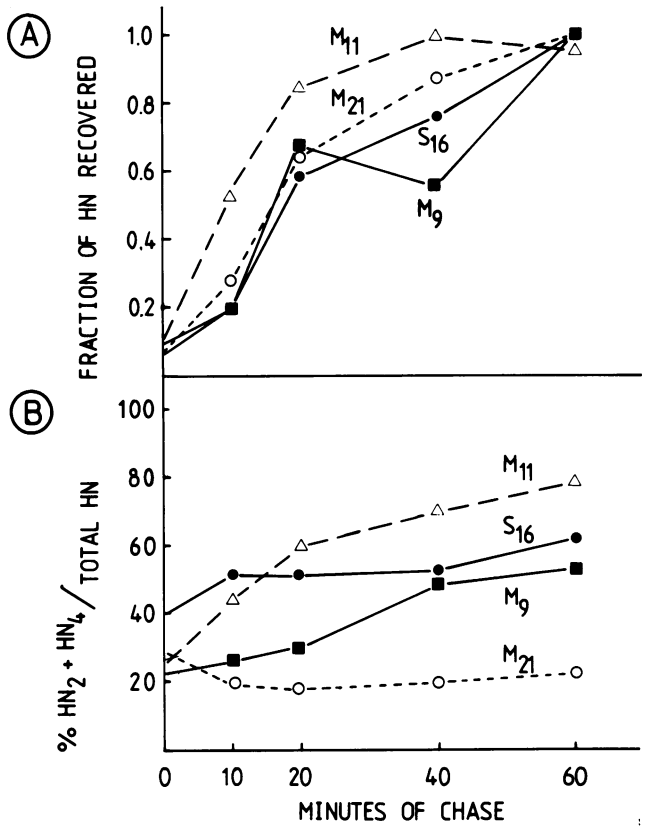

FIG. 9. Relationship between HN immunoreactivity and HN self-association. The amounts of HN proteins recovered in immunoprecipitations (R samples), as well as the fractions of HN in the form of $(HN)_2$ and $(HN)_4$ (NR samples), were estimated (see Materials and Methods) from experiments described in the legend to Fig. 8 or from similar experiments with different monoclonal antibodies and plotted as a function of the chase periods. (A) Fractions of HN recovered in immunoprecipitations. (B) Fractions of HN in dimer and tetramer forms.

monoclonal antibodies and five antigenic variants of A/USSR/90/77 revealed no major antigenic differences between the glycosylated and nonglycosylated (TM treated) form of HA." On the other hand, Alexander and Elder (1) showed changes in the immunoreactivity of glycosylated and endo-F-deglycosylated Raucher murine leukemia and influenza virus proteins. They found that "the more native the glycoprotein immunogens, the more dramatic the carbohydrate influence." Finally, Skehel et al. (25) very clearly demonstrated that a carbohydrate chain on the Hong Kong influenza virus hemagglutinin inhibits recognition by a monoclonal antibody, which, on the other hand, reacts perfectly with the protein made in the presence of tunicamycin.

The use of tunicamycin may be an inadequate way to estimate the importance of glycosylation in the maturation of HN and F_0 immunoreactivities, because preventing the addition of high-mannose sugars favors protein degradation. Therefore, tunicamycin could prevent the proteins from entering pathways in which the immunoreactivity would subsequently mature (directing the proteins directly to the lysosomes would be the most drastic). It is in fact known that HN_{TM} and F_{0TM} are not expressed at the cell surface (15; L. Roux, unpublished data); this lack of cell surface expression (of $F_{0_{TM}}$ in particular, which under normal conditions is found partly expressed at the cell surface immediately after its synthesis [4]) seems indeed to indicate that, in the presence of tunicamycin, HN and F_0 are never in the right pathway to normally mature. It is noteworthy that Sendai virus glycoproteins differ in that respect from influenza virus hemagglutinin, which was found expressed at the cell surface in the presence of tunicamycin (2).

Despite the uncertainty about the tunicamycin effect, it is clear that addition of high-mannose sugars is not sufficient to define HN native mature immunoreactivity, which needs a further maturation step. Two possibilities were therefore considered to account for this further step in maturation: (i) processing of high-mannose sugars and (ii) homopolymerization. Our results showed that although sugar processing took place during HN immunoreactivity maturation, it was an unlikely explanation for the maturation because the digestion of HN with endo-F had no effect on the degree of immunoreactivity. Endo-F removes all the N-linked sugar residues, regardless of their composition, provided that they are accessible to the enzyme; total accessibility is normally achieved by SDS denaturation of the protein before digestion (7). However, in our case, because SDS denaturation destroys the immunoreactivity, only the nondenatured protein was digested. It could then be argued that the critical sugars preventing the maturation of the antigenic sites were not removed by endo-F. However, if removal of sugar residues was important for the maturation of immunoreactivity, it would be expected that the sugars involved would be accessible to the processing enzymes and, therefore, to endo-F. Thus, the absence of an effect of endo-F digestion on the maturation of HN immunoreactivity, as well as on mature HN immunoreactivity, suggests that sugar residues are not directly involved in the modeling of HN immunoreactivity. This result was not totally unexpected; if addition of the bulky high-mannose sugars did not appear to define

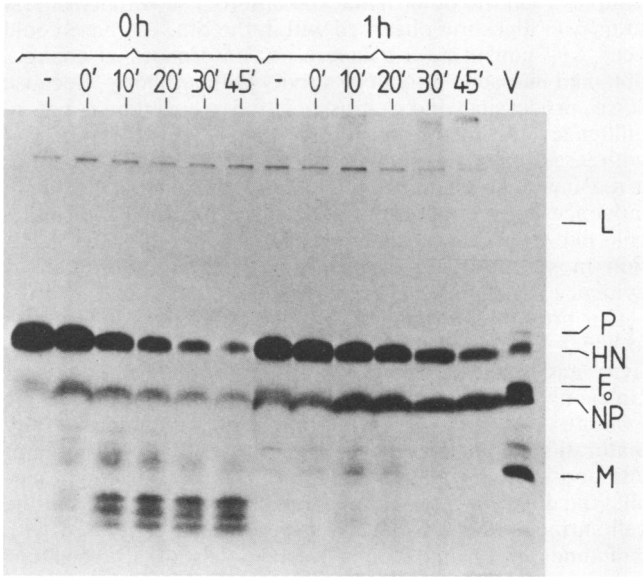

FIG. 10. HN sensitivity to trypsin digestion. Cytoplasmic extracts of pulse-labeled and chased infected cells were digested with 0.7 μg of trypsin for increasing periods of time, as described in Materials and Methods. After SDS denaturation of the cytoplasmic extracts, the HN protein and its degradation products were recovered by total cell immunoprecipitation with $RAb\text{-}HN_{SDS}$ and analyzed by PAGE. Abbreviations are defined in the legend to Fig. 1. —, Lanes with no trysin treatment.

the immunoreactivity, then minor changes in subsequent processing seemed unlikely to be important.

We expected that the homopolymerization of HN would be more important in defining its immunoreactivity because it could create new tertiary structures resulting from the self-association. The partial or total absence of correlation between the degree of self-association and the maturation of the HN immunoreactivity was therefore quite surprising, but it convincingly ruled out the direct participation of self-association in HN immunoreactivity maturation. The formation of at least part of the homodimers and tetramers appeared to start very early after HN synthesis and to continue while the protein was maturing and moving to the plasma membrane. It presumably goes on once HN is exposed at the cell surface, because in one comparison experiment (results not shown), HN at the cell surface was shown to exhibit a degree of polymerization lower than that of HN isolated from virus particles. This pattern of Sendai virus HN self-association correlates with that described by Schwalbe and Hightower (24) for the HN of Newcastle disease virus.

Although the obvious explanations for the slow maturation of HN native immunoreactivity were not obtained, we propose that our results evidence a slow maturation process of HN immunoreactivity via conformational changes. They cannot indeed simply reflect variations in accessibility of HN to antibody, on the hypothesis that HN would be trapped inside nondisrupted vesicles after the radioactive pulse and freed later on, because the solubilization of cellular extracts appeared complete as far as F_0 was concerned, even when F_0 was in the rough ER, as assessed by its complete sensitivity to endo-H. Moreover, HN immunoreactivity for an antiserum raised against the SDS-denatured HN did not increase with time under experimental conditions identical to those used to demonstrate an increase in reactivity for antibodies raised against the native protein (Fig. 2C, non-SDS-treated samples). On the other hand, the different sensitivity of HN to trypsin digestion observed within the time of chase could very well constitute a measure of conformational changes obtained independently of reactivity with antibody. Because sugar processing and self-association were shown not to influence HN immunoreactivity, that is, reactivity of HN with large molecules (molecular weight of about 150,000), it is reasonable to assume that these two phenomena would not influence the interaction of HN with a much smaller molecule like trypsin (molecular weight of about 20,000). Variation in sensitivity to trypsin can then be interpreted as evidence for changes in conformation.

Our present experiments revealed differences in the rates at which the two proteins acquire their native immunoreactivity and suggested that HN pauses in the ER or the *cis* Golgi apparatus (at least before the block in sugar processing exhibited by Ric^R 14 cells) during its immunoreactivity maturation (half time, 20 to 30 min). The different rates of immunoreactivity maturation for HN and F_0 strikingly parallel the different rates of appearance of these proteins at the cell surface, where F_0 and HN were found expressed with half times of 15 and 45 min, respectively (4). Such differences in the rates of cell surface expression have been reported for the G protein of vesicular stomatitis virus and the Newcastle disease virus HN protein (respective half times of appearance at the cell surface of 27 and 78 min [14]). The slow appearance of Newcastle disease virus HN at the cell surface resulted, as it did for Sendai virus HN, from a slow transit from the ER to the Golgi apparatus. Therefore, both HN proteins travel with similarly slow rates to the cell surface and are distinct in that respect from vesicular stomatitis virus G protein and Sendai virus F_0. Interestingly, both HN proteins are anchored in the membrane by their N termini (4, 9, 23), whereas vesicular stomatitis virus G protein and Sendai virus F_0 are anchored by their C termini (5, 18). Such opposite orientations in plasma membrane anchorage presumably implies differences in the mechanisms of insertion into the ER, as discussed by Engelman and Steitz (8). A protein with an N-terminus anchor has both ends attached to the ER membrane until the C terminus is completed and released free into the lumen. This is not the case for the C-terminus-anchored protein. The N terminus is released into the lumen as soon as the nascent chain has traversed the ER membrane and has its signal peptide cleaved; here, the folding of the polypeptide takes place freely on the growing chain and can be achieved by completion of protein synthesis. In the former case, it can reasonably be assumed that the folding of the polypeptide would be seriously constrained until the C terminus (the only free end) was released into the lumen. If the release of the C terminus into the lumen occurs slowly, it could constitute the limiting step in the proper folding of the HN protein. This would then account both for the delay of the protein in traversing the ER and the Golgi apparatus and for the lag observed in the immunoreactivity maturation. The reason for such putative slow release of the C terminus from the ER membrane would still have to be defined because a slow rate of HN protein synthesis is not observed. In addition, such a putative C terminus-membrane association would have to be transient.

Further studies on the immunoreactivity maturation of vesicular stomatitis virus protein G and Newcastle disease virus HN, as well as comparative studies on influenza virus hemagglutinin and neuraminidase, which are also anchored in different orientations (C and N terminus, respectively [3, 16, 26]) in the membrane, may prove to be useful.

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