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Intracellular Maturation of Mumps Virus Hemagglutinin-Neuraminidase Glycoprotein: Conformational Changes Detected with Monoclonal Antibodies

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Monoclonal antibodies elicited by immunization with mumps virus glycoproteins were selected with either native or chymotrypsin-treated mumps virus in an enzyme-linked immunosorbent assay. Group I antibodies which preferentially recognized chymotrypsin-treated virus failed to recognize native mumps virus hemagglutinin-neuraminidase (HN). They did react with sodium dodecyl sulfate-denatured HN and the HN chymotryptic fragments HNc2′ (molecular weight, 41,000) and HNc1 (molecular weight, 32,000) after transfer to nitrocellulose paper. In contrast, group II antibodies, which preferentially recognized native virus in the enzyme-linked immunosorbent assay, reacted with native HN but failed to bind HN after sodium dodecyl sulfate denaturation. These two groups of monoclonal antibodies were used to define the maturation pathway of the mumps virus HN in infected cells. The HN initially appeared as a 76,000-molecular-weight polypeptide and was recognized only by group I antibodies. A truncated form of HN, HN_T (molecular weight, 63,000), was synthesized in the presence of tunicamycin and was also recognized only by group I antibodies. The 76,000-molecular-weight HN was rapidly converted to a 74,000-molecular-weight polypeptide; this form of HN was recognized only by group II antibodies. The oligosaccharide side chains were modified, and intermolecular disulfide bonds were formed as HN was transported to the cell surface. The disulfide-linked oligomers of HN were direct precursors of the HN found in mature virus.

The eucaryotic cell has a highly organized system of subcellular compartments that are responsible for the post-translational processing of secretory and membrane-bound proteins. These proteins are first synthesized by ribosomes bound to the rough endoplasmic reticulum (RER), and as they enter the lumen of this compartment, they encounter enzymes that begin to modify the protein (10, 14). Signal peptidase may remove the amino-terminal signal peptide as a cotranslational event (5), and frequently simple-type oligosaccharide side chains (high-mannose type) are cotranslationally added to asparagine residues (14). Glucose residues are then removed from the high-mannose oligosaccharide side chain by a glucosidase (10), and recent evidence suggests that disulfide bond formation also occurs in the RER (28).

The proteins then continue their maturation in the Golgi apparatus of the cell. The exact mechanism of protein transport from the RER to the Golgi apparatus is unknown; however, transport vesicles that bud from the RER and then fuse with the Golgi apparatus have been described previously (18, 20). The Golgi appartus has been separated into the *cis*-Golgi and the *trans*-Golgi on the basis of the types of enzymes found in these two compartments (4, 19). It appears that maturing proteins first encounter the *cis*-Golgi (4, 19). There, terminal mannose residues are removed from the oligosaccharide side chain by mannosidases I and II, and *N*-acetylglucosamine residues are added by the action of *N*-acetylglucosamine transferases I and II (3). The protein is then transported to the *trans*-Golgi, where galactosyl transferase, fucosyl transferase, and sialyl transferase add their sugar substrates to the maturing oligosaccharide side chains (3, 4). The *trans*-Golgi also appears to contain enzymes responsible for the sulfation and selective proteolysis of certain proteins (19). The secretory or plasma membrane glycoprotein is then transported to its final destination, presumably by a vesicle transport system similar to the one that shuttles proteins from the RER to the Golgi (25).

Many of these posttranslational processing events have been elucidated by studying the maturation of viral glycoproteins, in particular the vesicular stomatitis virus G protein (1, 4, 25, 30). In this report, we describe the use of the mumps virus hemagglutinin-neuraminidase (HN) to follow the processing steps that this integral membrane glycoprotein encounters. We demonstrate that conformational changes occur in HN as it is enzymatically processed and also define some of the structural differences in the HN glycoprotein that appear necessary for its modification. These studies were facilitated by using monoclonal antibodies that recognize distinct conformations of the HN glycoprotein.

MATERIALS AND METHODS

Cells and virus. The plaque-purified O'Take strain of mumps virus was grown in CV-1 cells and purified as described previously (23). Virus was radiolabeled with [4,5-^{3}H]leucine (58 Ci/mmol; ICN Pharmaceuticals Inc., Irvine, Calif.) or [^{35}S]methionine (1,100 to 1,300 Ci/mmol; Amersham Corp., Arlington Heights, Ill.) as described previously (23). Purified virus was stored at −80°C before use.

Tunicamycin treatment and preparation of infected cell lysates. O'Take virus-infected CV-1 cells were treated at 24 h after infection with 5 μg of tunicamycin (Calbiochem-Behring, La Jolla, Calif.) per ml for 30 min at 37°C in minimum essential medium deficient in the amino acid leucine. The cells were then labeled with 50 μCi of

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[^{3}H]leucine per ml in leucine-deficient minimum essential medium containing 5 μg of tunicamycin per ml for 2 h at 37°C. The monolayers were washed three times with ice-cold 0.01 M Tris hydrochloride [pH 7.2] containing 0.15 M NaCl and then disrupted by the addition of lysate buffer (23). The DNA was sheared by passing the lysates through a 23-gauge needle five times, and the lysates were clarified by centrifugation at 100,000 × *g* for 1 h just before use in immunoprecipitation assays. Non-tunicamycin-treated infected cell lysates and uninfected cell lysates were prepared in an otherwise identical fashion and were radiolabeled with 50 μCi of [^{3}H]leucine, D-[2-^{3}H]mannose (20 Ci/mmol; ICN), or L-[6-^{3}H]fucose (30 Ci/mmol; ICN) per ml.

Pulse and pulse-chase analysis. At 24 h postinfection, O'Take-infected CV-1 cells were incubated in minimum essential medium deficient in methionine for 30 min at 37°C. Identical medium containing 50 μCi of [^{35}S]methionine per ml was then added to the monolayers for 15 min at 37°C. The medium was then replaced with minimum essential medium containing 10 times the normal concentration of methionine, and the cells were incubated at 37°C for 15, 30, 60, or 180 min. The individual monolayers were then prepared for immunoprecipitation assays as described above.

Cell surface immunoprecipitation analysis. At 24 h postinfection, CV-1 cells were radiolabeled with [^{35}S]methionine for 2 h. The cells were then washed three times with ice-cold Hanks balanced salt solution containing 0.1% bovine serum albumin, and prechilled antibodies diluted in this same buffer were permitted to bind to the monolayers for 30 min while incubating on ice. The monolayers were then washed five times with this same buffer and processed as the antigen for immunoprecipitation assays.

Endoglycosidase H digestion. Immunoprecipitation assays were performed with monoclonal antibodies and [^{35}S]methionine-labeled infected-cell lysate as antigen. At the end of the assay, the immune complexes were suspended in 1% sodium dodecyl sulfate (SDS) and 0.1 M Tris (pH 8.0), and then 2-mercaptoethanol was added to a 1% final concentration. The samples were boiled for 10 min and pelleted, and the supernatant was made 0.25 M iodoacetamide with a fresh stock of 1.0 M iodoacetamide. After 30 min, each sample was precipitated with 10% trichloroacetic acid for 1 h on ice and pelleted, and each pellet was washed with acetone at −20°C. Each pellet was redissolved in 0.3 M sodium citrate (pH 6.0) containing 0.5% SDS and 1 mM phenylmethylsulfonyl fluoride. Each sample was then divided in half, and one half received 4 μl of endoglycosidase H (45 μg/ml; NENzymes, Boston, Mass.). The other half of the sample received buffer without enzyme. All samples were incubated at 37°C for 3 h, then an additional 2 μl of endoglycosidase H was added to the endoglycosidase H-containing tube. After 4 additional h at 37°C, the reactions were terminated, and the samples were precipitated by the addition of 5 volumes of −20°C acetone. The precipitates were collected by centrifugation, and the pellets were suspended in SDS-sample buffer for analysis.

Antibodies. Monoclonal antibodies were produced and characterized as described previously (29). Mice used as spleen donors were immunized with O'Take virus glycoproteins extracted from virions with the nonionic detergent EMBC-720 (Sigma Chemical Co., St. Louis, Mo.) and emulsified in complete Freund adjuvant. Booster immunizations were repeated at biweekly intervals, with the same glycoprotein extract in incomplete Freund adjuvant. An enzyme-linked immunosorbent assay (ELISA) was performed on wells having positive fusion events (29). The antigen for this ELISA was either sucrose step gradient-purified O'Take virus or the same purified virus treated for 2 h with chymotrypsin (16). The hyperimmune rabbit anti-mumps serum was prepared against the Enders strain of mumps virus; its characterization has been detailed previously (15, 23).

Immunoprecipitation and SDS-PAGE. Radiolabeled virus antigen was prepared by disrupting purified virus in lysate buffer for 1 h at 4°C. The lysate was then clarified at 10,000 × *g* for 5 min just before its use in immunoprecipitation assays. Radiolabeled infected cell antigens were prepared as described above. Immunoprecipitation assays were accomplished with protein A-Sepharose CL-4B (Pharmacia, Inc., Piscataway, N.J.) as the solid-phase adsorbent (23). SDS-polyacrylamide gel electrophoresis (PAGE) was performed with the discontinuous buffer system of Laemmli (12). Fluorography was done with XAR-5 X-ray film (Eastman Kodak Co., Rochester, N.Y.).

Immunoblotting. The electrophoretic transfer of mumps virus polypeptides to nitrocellulose (NC) paper (BA83; Schleicher & Schuell, Inc., Keene, N. H.) was accomplished with a Transblot apparatus (Bio-Rad Laboratories, Richmond, Calif.) set at 50 V for 4 h. The transfer buffer was 25 mM Tris hydrochloride–192 mM glycine–0.02% SDS–20% methanol. The mumps virus polypeptides were detected with monoclonal antibodies in ascites fluid diluted 1:500. The bound monoclonal antibodies were detected and visualized with a Vectastain ABC kit (Vector Laboratories, Inc., Burlingame, Calif.). Diaminobenzidine tetrahydrochloride served as the peroxidase substrate in the final step, which produced the reaction product.

Immunofluorescence. CV-1 cells were grown in 8-well chamber slides (Miles Scientific, Div. Miles Laboratories, Inc., Naperville, Ill.) and infected as usual. After 24 h, the cells were washed and then fixed with 95% ethanol at −20°C, absolute methanol at −20°C, or 10% Formalin at 4°C. Some of the Formalin-fixed monolayers were then treated for 15 min with 1% Triton X-100 in phosphate-buffered saline to permeabilize the cell membranes. The monolayers were then washed with phosphate-buffered saline and stained by adding ascites fluid containing monoclonal antibodies diluted 1:200 in phosphate-buffered saline with 0.5% bovine serum albumin. After 30 min, the monolayers were washed and then incubated in biotinylated goat anti-mouse immunoglobulin G (IgG) (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) for 30 min. After being washed, streptavidin-Texas Red was added to the monolayers for 30 min. The slides were then washed and mounted in Aquafluor (Lerner Laboratories, New Haven, Conn.) and observed for immunofluorescence. All photomicrographs were exposed for the same period of time.

RESULTS

Production, screening, and characterization of mumps virus glycoprotein-specific antibodies. BALB/c mice were hyperimmunized with mumps virus glycoproteins extracted from purified virus with the nonionic detergent EMBC-720. The immune spleen cells from these animals were fused with the NS-1 myeloma cell line, and the supernatants from the resulting hybridomas were assayed in an ELISA system with either purified virus or chymotrypsin-treated purified virus as the antigen. A total of 17 monoclonal antibodies were identified as mumps virus specific by their ability to bind in this assay. One group of six antibodies (group I) showed a two- to fourfold enhancement in ability to bind antigen after it was treated with chymotrypsin. A second group of seven

antibodies (group II) exhibited 2- to 10-fold less binding when the virus antigen was treated with chymotrypsin. A third group of four antibodies (group III) was identified that showed smaller changes in the differential ability to bind either untreated or chymotrypsin-treated antigen. Three members of each group were selected for further analysis, and, except where noted, the reactivity of the prototype selected from each group is representative of each member in that group.

The antigen specificity of each monoclonal antibody was initially determined by immunoprecipitation assays using [^{3}H]leucine-labeled mumps virus disrupted in lysate buffer as the antigen, followed by SDS-PAGE. Group I monoclonal antibodies did not immunoprecipitate any polypeptide from lysates of [^{3}H]leucine-labeled virus (Fig. 1, lanes b and e). Group II monoclonal antibodies immunoprecipitated a 74,000-molecular-weight polypeptide when analyzed with reduced disulfide bonds (Fig. 1, lane c) or as a 160,000-molecular-weight polypeptide with intact disulfide bonds (Fig. 1, lane f). A portion of the disrupted [^{3}H]leucine-labeled mumps virus used for the immunoprecipitation assays was also analyzed by SDS-PAGE with or without the reduction of disulfide bonds (Fig. 1, lanes a and d, respectively). The polypeptides visible in lane a of Fig. 1 are labeled L, HN, NP, F_1, *F_1, P, and M, respectively, in order of decreasing molecular weight, as defined previously (15).

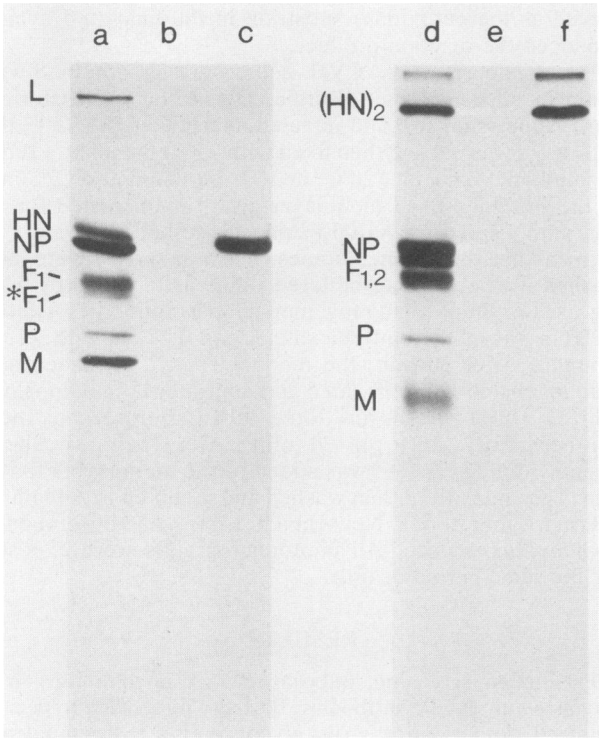

FIG. 1. Antigenic reactivity of group I and group II monoclonal antibodies with mumps virus. A clarified lysate of purified mumps virus labeled with [^{3}H]leucine was analyzed by SDS-PAGE in the presence (lane a) or absence (lane d) of dithiothreitol. A portion of this virus preparation was used as the antigen for immunoprecipitation assays with group I antibodies (lanes b and e) or group II antibodies (lanes c and f). Lanes b and c represent SDS-PAGE analysis in the presence of dithiothreitol. Lanes e and f were analyzed without dithiothreitol. Each assay was performed with equal amounts of radiolabeled antigen. The polypeptide designations are described in Results.

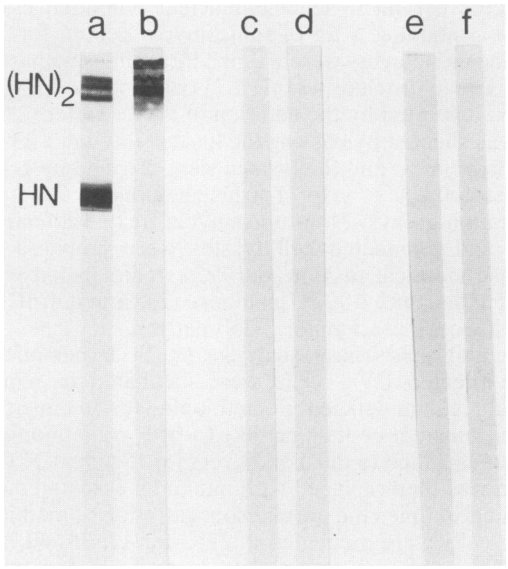

FIG. 2. Antigenic reactivity of group I and group II monoclonal antibodies with mumps virus polypeptides transferred from SDS-PAGE to NC paper. A 10-μg amount of mumps virus was disrupted in SDS sample buffer with or without dithiothreitol and electrophoresed on SDS-PAGE. The polypeptides were then electrophoretically transferred to NC paper and stained immunologically. Lanes a and b were stained with group I monoclonal antibody, lanes c and d were stained with group II monoclonal antibody, and lanes e and f represent the staining when no primary antibody was added to the reaction mixture. Lanes a, c, and e were from an SDS-PAGE with the addition of dithiothreitol; lanes b, d, and f were from an SDS-PAGE without dithiothreitol. The reaction product was developed as described in Materials and Methods.

The F_2 polypeptide is not identified in this figure. The unreduced polypeptides are labeled $(HN)_2$, NP, $F_{1,2}$, P, and M; $F_{1,2}$ represents both $F_{1,2}$ and *$F_{1,2}$ which did not separate well in this SDS-PAGE. The NP polypeptide appears as two separable bands when analyzed unreduced (Fig. 1, lane d). The identification of each mumps virus polypeptide without reduction of disulfide bonds was accomplished by separate immunoprecipitation assays using monoclonal antibodies to each of the specified polypeptides (data not shown). Group III monoclonal antibodies immunoprecipitated the mumps virus fusion protein; they will not be discussed further in this report.

Group I and group II monoclonal antibodies were then tested for their ability to bind mumps virus polypeptides electrophoretically transferred from SDS-PAGE to NC paper. Group I antibodies, which did not bind to HN in lysates of mumps virus, bound specifically to the HN polypeptide transferred to NC paper after electrophoresis (Fig. 2, lane a). Group I monoclonal antibodies also bound to the $(HN)_2$ polypeptide identified when mumps virus was analyzed by SDS-PAGE without the reduction of disulfide bonds (Fig. 2, lane b). The group II monoclonal antibodies, which specifically bound to HN in lysates of purified mumps virus, did not bind to HN after it was transferred to NC paper with or without the reduction of disulfide bonds (Fig. 2, lanes c and d, respectively). Minimal staining was seen when no primary antibody was added to the assay (Fig. 2, lanes e and f).

Group I antibodies were initially selected on the basis of increased binding to chymotrypsin-treated virus. To better define what these antibodies recognized, we took advantage

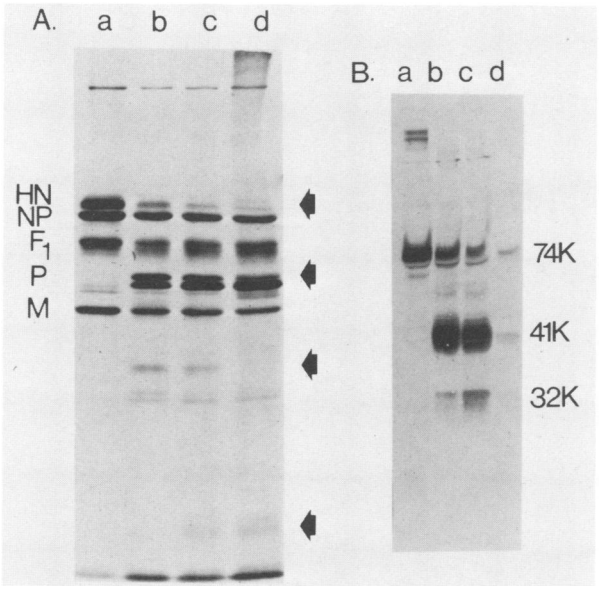

FIG. 3. SDS-PAGE analysis of mumps virus treated with chymotrypsin. Mumps virus was treated with chymotrypsin for 0, 30, 60, or 180 min. After the reaction was stopped with aprotinin, the digested peptides were removed from the virus particles by ultracentrifugation through a layer of 20% glycerol containing aprotinin. The virus was resuspended in SDS sample buffer and analyzed by SDS-PAGE. (A) Lanes a, b, c, and d show the virus polypeptides remaining with the virus particle after 0, 30, 60, or 180 min of chymotrypsin digestion, respectively. The arrowheads indicate the HN-related polypeptides. (B) A second set of lanes from the same SDS-PAGE was transferred to NC paper as described above and stained with group I monoclonal antibodies. Lanes a, b, c, and d show the immunologic identification of the HN-related chymotryptic fragments after 0, 30, 60, or 180 min of chymotrypsin digestion, respectively. The approximate molecular weights of the major HN-reactive polypeptides are indicated to the right of Panel B.

of their ability to bind denatured HN antigenic determinants. The reactivity pattern of group I antibodies when SDS-denatured chymotrypsin-treated mumps virus polypeptides were used in immunoblot analysis is shown in Fig. 3. Panel A shows the selective sequential degradation of mumps virus HN by chymotrypsin. HN was initially cleaved to yield HNc2′ (molecular weight, 41,000) and HNc1 (molecular weight, 32,000). After 180 min, HNc1 and HNc2′ were degraded, leaving the membrane-associated HNc2 (molecular weight, 19,000) polypeptide. Panel B depicts a second set of gel lanes transferred to nitrocellulose paper and then stained with a group I antibody. In addition to recognizing the HN polypeptide, group I antibodies recognized HNc2′ and, to a lesser extent, HNc1; HNc2 was never recognized.

Maturation of mumps virus HN in infected cells. Infected cells were pulse-labeled for 15 min, and the radiolabeled polypeptides were chased for the times indicated in the legend to Fig. 4. This SDS-PAGE analysis was done without the reduction of disulfide bonds. Group I antibodies immunoprecipitated a protein that electrophoresed as a broad band with an average molecular weight of 76,000 (Fig. 4; lanes a to e). The maximum amount of this radiolabeled polypeptide was immunoprecipitated from cells after a 15-min chase period (Fig. 4, lane b) and subsequently decreased with time. Very little of the 76,000-molecular-weight polypeptide was detected after a 3-h chase period (Fig. 4, lane e). Group II antibodies immunoprecipitated a 74,000-molecular-weight polypeptide after the 15-min pulse (Fig. 4, lane f); more of this polypeptide was seen after a 15-min chase period (Fig. 4, lane g). This 74,000-molecular-weight polypeptide was slightly smaller than the 76,000-molecular-weight polypeptide immunoprecipitated by group I antibodies; it also exhibited a less heterogeneous molecular weight (compare lanes b and g of Fig. 4). After a 30-min chase period, a second polypeptide appeared in infected cells that was recognized by group II antibodies (Fig. 4, lane h). In the figure, this polypeptide was labeled $(HN)_2$ and had an approximate molecular weight of 160,000. Progressively less of the 74,000-molecular-weight polypeptide was detected as more of the 160,000-molecular-weight polypeptide appeared (Fig. 4, lane i). After a 3-h chase period, very little of the 74,000-molecular-weight poypeptide was present, whereas an intense 160,000-molecular-weight band was present (Fig. 4, lane j). Of note was that neither group I nor group II antibodies recognized any host cell protein in infected or uninfected cells or the F_1 or F_2 polypeptides in mumps virus-infected cells.

Densitometric tracings of each gel lane indicated that the half-life of the 76,000-molecular-weight HN molecule was approximately 25 min. This half-life was calculated from the time the radiolabeled [^{35}S]methionine was first added to the cell. The 74,000-molecular-weight form of HN attained a maximum level at approximately 60 min. The half-life of this molecule was difficult to determine from densitometric tracings because the intracellular pool of this molecule was determined by both maturation from radiolabeled precursors and maturation into the 160,000-molecular-weight HN. Almost the entire pool of the 74,000-molecular-weight HN had

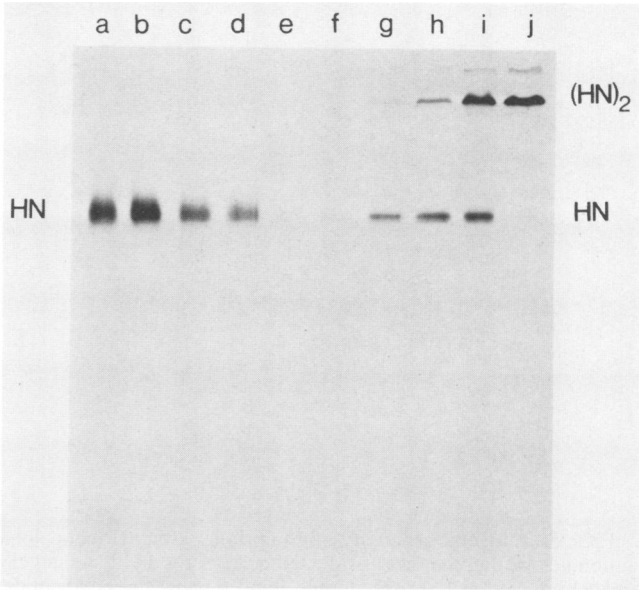

FIG. 4. Kinetic analysis of mumps virus HN glycoprotein in infected cells by selective immunoprecipitation. Infected cells were pulse-labeled for 15 min with [^{35}S]methionine. Infected cell lysates used in these immunoprecipitation assays were obtained after chase periods of 0 (lanes a and f), 15 (lanes b and g), 30 (lanes c and h), 60 (lanes d and i), and 180 min (lanes e and j). Lanes a to e show the results with a group I antibody; lanes f to j show the results with a group II antibody. Portions of infected cell lysates used for each immunoprecipitation contained material derived from an equivalent number of cells. Polypeptide designations are described in Results.

matured into the 160,000-molecular-weight HN by 3 h after the label was first added to the cells.

Effect of tunicamycin treatment on the HN glycoprotein. We were interested in studying what posttranslational modifications could produce the two conformations of the HN glycoprotein identified with monoclonal antibodies. One modification is the addition of oligosaccharides to a protein. The glycosylation of proteins at asparagine residues can be inhibited by treating cells with the drug tunicamycin. Tunicamycin prevents the formation of an intermediate that is necessary for the transfer of oligosaccharides to proteins (26). We inhibited the glycosylation of mumps virus proteins by treating infected cells with tunicamycin for 30 min before radiolabeling the cells with [^{3}H]leucine. The hyperimmune anti-mumps rabbit serum was used to analyze the spectrum of mumps virus-specific polypeptides labeled in infected cells with or without treatment with tunicamycin. All of the mumps virus structural polypeptides were immunoprecipitated when cells were labeled in the absence of tunicamycin (Fig. 5, lane a). In the presence of tunicamycin, the mumps virus antiserum immunoprecipitated L (not seen well in this figure), NP, P, and M, but no polypeptides corresponding to HN or F were visible (compare Fig. 5, lanes a and b). A longer exposure of lane b revealed three new approximately 63,000-, 51,000-, and 48,000-molecular-weight species (data not shown). The small amounts of the unglycosylated forms of the HN and F polypeptides probably reflects the instability of these aberrant polypeptides in the infected cell or a lack of antibodies in the hyperimmune serum capable of recognizing these polypeptides or both.

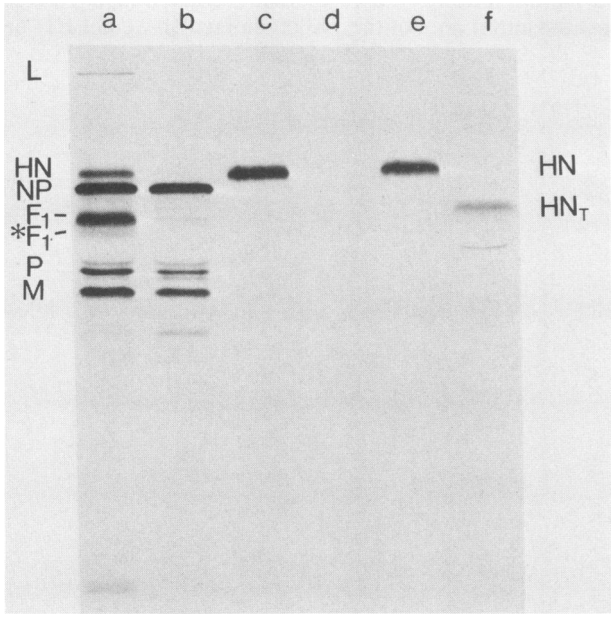

FIG. 5. Antigenic reactivity of group I and group II monoclonal antibodies with mumps virus-infected cell lysates and tunicamycin-treated infected-cell lysates. Lanes a and b show the polypeptides immunoprecipitated by hyperimmune rabbit anti-mumps serum. Lanes c and d show the polypeptides immunoprecipitated by group II antibodies. Lanes e and f show the polypeptides immunoprecipitated by group I antibodies. Lanes a, c, and e are the results with untreated infected cell lysates as the antigen. Lanes b, d, and f are the results with tunicamycin-treated infected cell lysates as the antigen. The polypeptides in each instance were labeled with [^{3}H]leucine. Portions of infected cell lysates used for each immunoprecipitation contained material derived from equivalent numbers of cells. The polypeptide designations are described in Results.

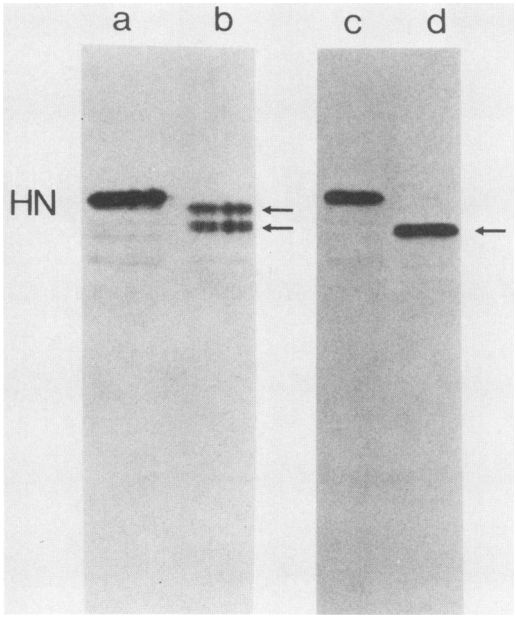

FIG. 6. SDS-PAGE of the endoglycosidase H-digested polypeptides immunoprecipitated with group I and group II monoclonal antibodies. Group I and group II monoclonal antibodies were used in immunoprecipitation assays using [^{35}S]methionine-labeled mumps virus-infected cell lysates as the antigen. Each sample was then divided into two, and one-half of each sample was digested with endoglycosidase H. Lane a shows the undigested half of the sample immunoprecipitated with group II monoclonal antibodies; lane b shows the digested half of the sample. Lane c shows the undigested half of the sample immunoprecipitated with group I monoclonal antibodies; lane d shows the digested half of the sample. The arrows indicate the major polypeptides produced by digestion with endoglycosidase H.

An analysis of immunoprecipitation assays using group II antibodies (Fig. 5, lanes c and d) identified the 74,000-molecular-weight band as the HN glycopolypeptide in untreated infected-cell lysates (Fig. 5, lane c). However, no polypeptide was detected when tunicamycin-treated infected cells were used as the antigen (Fig. 5, lane d). The group I antibodies immunoprecipitated the 76,000-molecular-weight band in untreated infected cells (Fig. 5, lane e). Group I antibodies also reacted with the 63,000-molecular-weight polypeptide in tunicamycin-treated infected cells (Fig. 5, lane f) and identified it as an unglycosylated precursor of the HN glycoprotein. This polypeptide was labeled HN_T, consistent with the nomenclature used by Herrler and Compans (8). None of these monoclonal antibodies immunoprecipitated polypeptides from uninfected CV-1 cell lysates prepared in an identical fashion (data not shown).

Endoglycosidase H digestion of HN. The polypeptides immunoprecipitated by group I and group II monoclonal antibodies were digested with endoglycosidase H as described above. Endoglycosidase H removes the *N*-acetylchitobiose core of high-mannose-type oligosaccharides, leaving complex oligosaccharide side chains intact (27). When the HN immunoprecipitated with the group II monoclonal antibodies (Fig. 6, lane a) was digested with endoglycosidase H, two new polypeptides could be identified, with approximate molecular weights of 69,000 and

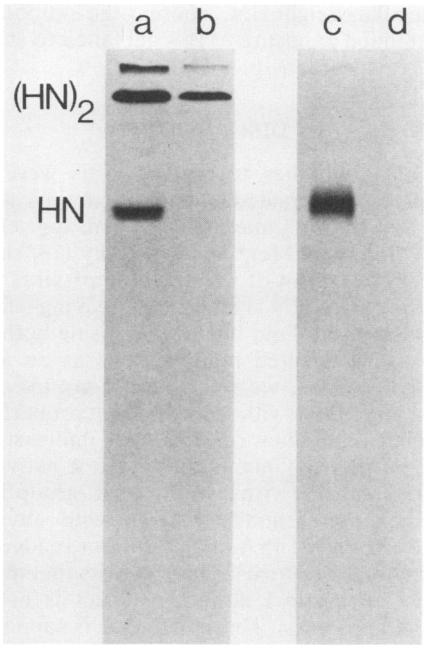

FIG. 7. SDS-PAGE of immunoprecipitation assays using group I and group II monoclonal antibodies and [^{3}H]mannose- or [^{3}H]fucose-labeled infected cell lysates. Lanes a and b show the radiolabeled polypeptides immunoprecipitated by group II monoclonal antibodies; lanes c and d show the polypeptides immunoprecipitated by group I monoclonal antibodies. The antigen in lanes a and c was [^{3}H]mannose-labeled infected-cell lysates; the antigen in lanes b and d was [^{3}H]fucose-labeled infected cell lysates.

64,000 when the products were analyzed by SDS-PAGE (see arrows in Fig. 6, lane b). When the HN immunoprecipitated by group I monoclonal antibodies (Fig. 6, lane c) was digested with endoglycosidase H, only a single polypeptide was identified, with an approximate molecular weight of 63,000 (see arrow in Fig. 6, lane d). Endoglycosidase H digestion of HN isolated from mature virus indicated that HN never becomes completely resistant to the action of this enzyme (data not shown).

Radiolabeling of HN with specific carbohydrates. Uninfected and mumps virus-infected cells were radiolabeled with either [^{3}H]mannose or [^{3}H]fucose, and lysates were prepared for immunoprecipitation assays. Group II monoclonal antibodies immunoprecipitated two glycopolypeptides from infected cells labeled with [^{3}H]mannose (Fig. 7, lane a): a 74,000-molecular-weight glycopolypeptide, labeled HN; and a 160,000-molecular-weight glycopolypeptide, labeled $(HN)_2$. When infected cells labeled with [^{3}H]fucose were used as the antigen for group II monoclonal antibodies, only the 160,000-molecular-weight glycopolypeptide was identified by SDS-PAGE (Fig. 7, lane b). In contrast, group I monoclonal antibodies immunoprecipitated a single 76,000-molecular-weight glycopolypeptide when infected cells were labeled with [^{3}H]mannose (Fig. 7, lane c) and no polypeptide when the infected cells were labeled with [^{3}H]fucose (Fig. 7, lane d). No polypeptides were immunoprecipitated from uninfected cells radiolabeled with these same carbohydrates (data not shown).

Intracellular distribution of HN. The distribution of HN was determined by an immunofluorescence assay on Formalin-fixed and Triton X-100-permeabilized mumps virus-infected cells. The immunofluorescence staining patterns

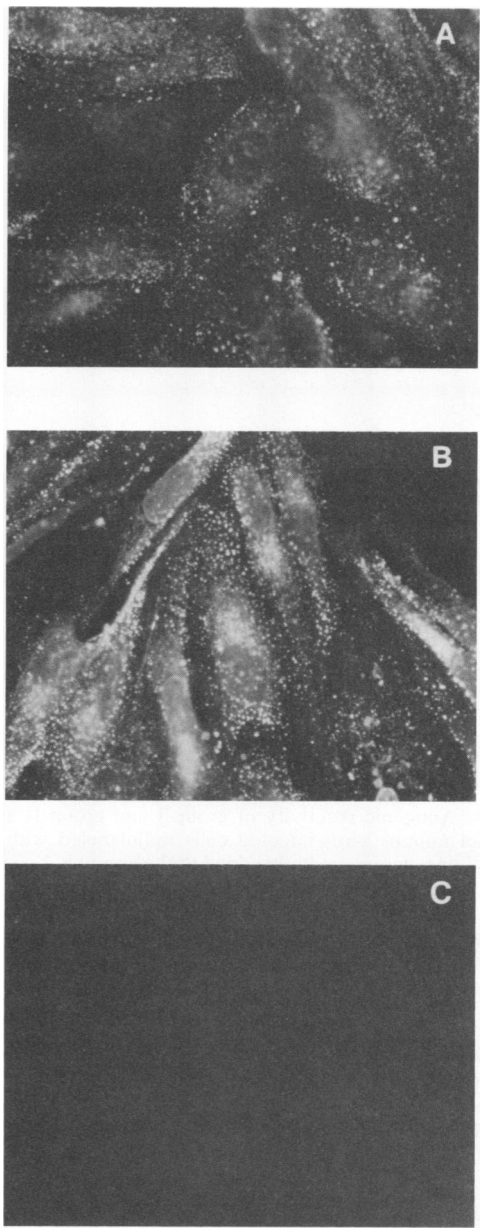

FIG. 8. Immunofluorescence assay of mumps virus-infected cells. Mumps virus-infected cells were fixed with Formalin and permeabilized with Triton X-100. Individual monolayers were then stained with group I monoclonal antibodies (A), group II monoclonal antibodies (B), or no primary antibody (C). The immunofluorescence was produced by streptavidin-Texas Red that was immunologically linked to the primary antibody by biotinylated goat anti-mouse IgG. Each photomicrograph was exposed for the same period of time.

with group I and group II monoclonal antibodies could not be distinguished from each other (compare Fig. 8, panels A and B). The pattern included punctate staining, which appeared to be on the surface of the cell, and a diffuse staining surrounding the cell nucleus. No staining was seen when antibody was not added to the monolayers (Fig. 8, panel C), nor was staining seen when uninfected cells were treated with these antibodies (data not shown). Primary fixation of infected cells with ethanol, methanol, or Formalin produced

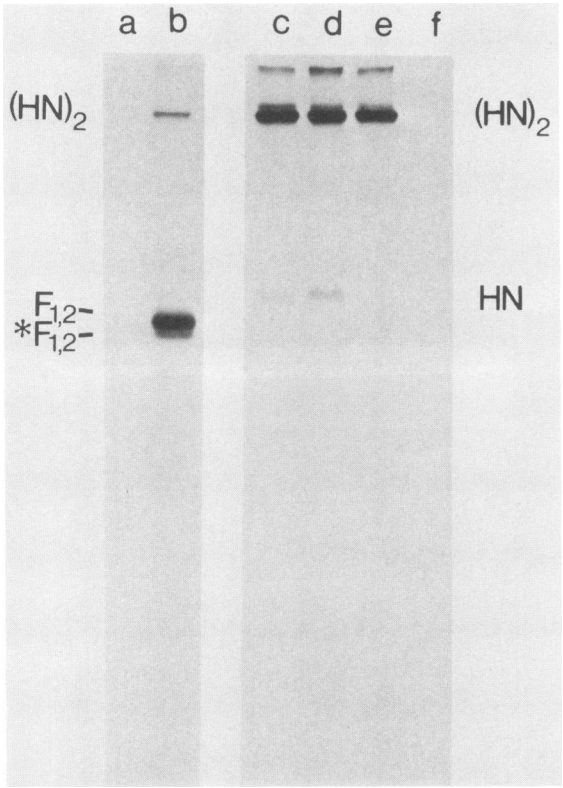

FIG. 9. Antigenic reactivity of group I and group II antibodies with intact mumps virus-infected cells radiolabeled with [^{35}S]methionine. Lanes: a, no antibody added to the assay; b, hyperimmune rabbit anti-mumps virus antibody added; c, d, and e, three different group II antibodies added; f, group I antibody added. An equal number of radiolabeled infected cells were used as the antigen in each assay. This SDS-PAGE analysis was done without dithiothreitol. The polypeptide designations are described in Results.

the same patterns when cells were reacted with either group I or group II monoclonal antibodies.

Presence of the HN molecule on the surfaces of infected cells. The preceding data indicated that three distinct forms of HN were found within mumps virus-infected cells. By exposing radiolabeled infected cell monolayers to antibodies before disrupting the cells, we determined which conformations of HN were transported to the cell surface. The hyperimmune rabbit anti-mumps serum recognized all of the structural proteins of mumps virus, including the internal polypeptides NP, P, and M (Fig. 9, lane a). When this hyperimmune serum was used to probe the surface of infected cells, it recognized three polypeptides, $(HN)_2$, $F_{1,2}$, and $*F_{1,2}$, when analyzed by SDS-PAGE without reducing agent (Fig. 9, lane b). This hyperimmune serum was previously shown to immunoprecipitate F_o from infected cell lysates (15). However, no F_o was detected on the surfaces of infected cells by this technique. No polypeptides were detected when primary antibody was deleted from the assay (Fig. 9, lane a).

Group I antibodies showed a complete lack of immunologic reactivity with the HN molecules located on the surfaces of infected cells (Fig. 9, lane f). The three group II antibodies showed two types of immunoprecipitation patterns. All three immunoprecipitated dimeric HN from the surfaces of infected cells (Fig. 9, lanes c, d, and e). However, the antibodies used in lanes c and d also recognized the 74,000-molecular-weight HN, whereas the antibody in lane e did not. Extended exposure of this gel failed to show a band in the HN region of lane e.

DISCUSSION

Monoclonal antibodies to mumps virus were produced with glycoproteins extracted from purified virus as immunizing agents and by a unique assay system for selecting the resulting hybridomas. Merz and Wolinsky (16) showed that chymotrypsin treatment of purified mumps virus removes a portion of the native HN glycoprotein, leaving a fragment of HN embedded in the lipid bilayer. By using both untreated and chymotrypsin-treated mumps virus as an antigen for screening hybridomas, we selected two groups of anti-HN monoclonal antibodies with very different properties. Group II monoclonal antibodies reacted with undigested mumps virus in the ELISA and immunoprecipitated native HN from lysates of radiolabeled virus. In contrast, group I monoclonal antibodies preferentially reacted with chymotrypsin-treated virus in the ELISA and could not recognize HN in lysates of radiolabeled virus. This suggests that the antigenic site with which group I antibodies react is not normally exposed in mumps virus. This conclusion is supported by the ability of group I antibodies to bind HN that is SDS denatured and transferred to NC paper. SDS treatment with or without the reduction of disulfide bonds was sufficient to allow the binding of group I monoclonal antibodies. In contrast, the group II monoclonal antibodies did not bind HN in this assay. These reactivity patterns are consistent with the concept of continuous and discontinuous antigenic sites. Group I monoclonal antibodies appear to recognize a linear sequence of amino acids (continuous site); group II monoclonal antibodies appear to recognize a sequence of amino acids that are dependent on a specific conformation (discontinuous site).

The group I monoclonal antibodies recognized both the 41,000- and 32,000-molecular-weight chymotryptic fragments of HN but were not reactive with the 19,000-molecular-weight-limit digest fragment. Merz and Wolinsky (16) suggested that the 41,000- and 32,000-molecular-weight fragments of HN were derived from separate areas of the HN molecule; however, the present data suggest that these two polypeptide fragments share a common antigenic site. Alternatively, the amino acids that produce the antigenic site that these antibodies recognized may have an internal chymotrypsin cleavage site. Chymotrypsin digestion would divide the antigenic site, with a portion being on the 41,000-molecular-weight fragment and a portion being on the 32,000-molecular-weight fragment. This hypothesis could explain why the 41,000-molecular-weight polypeptide reacted more strongly with group I antibodies than the 32,000-molecular-weight polypeptide if the 41,000-molecular-weight fragment contained a larger portion of the antigenic site.

We postulated that newly synthesized HN may not assume certain conformations until it is posttranslationally processed into its mature form. This was confirmed by probing radiolabeled lysates of infected cells with group I and group II monoclonal antibodies. Group I monoclonal antibodies bound to a 76,000-molecular-weight polypeptide in infected cells that rapidly disappeared. As this 76,000-molecular-weight polypeptide disappeared, group II monoclonal antibodies began to recognize a 74,000-molecular-weight polypeptide; as this disappeared, a 160,000-molecular-weight polypeptide appeared that was also recognized by group II monoclonal antibodies.

These monoclonal antibodies also served as probes for determining the biochemical differences between the distinct HN polypeptides. For instance, by completely blocking the normal glycosylation of proteins with tunicamycin, we found that only group I monoclonal antibodies could react with an unglycosylated 63,000-molecular-weight form of the HN protein. Group II antibodies did not bind HN when the protein was not glycosylated, indicating that N-linked glycosylation is required for HN to assume a conformation recognized by group II monoclonal antibodies.

The endoglycosidase H digestion studies suggest that all of the oligosaccharide side chains on the 76,000-molecular-weight HN molecule are of the high-mannose type, consistent with the first step in the N-linked glycosylation of proteins. The 63,000-molecular-weight polypeptide produced by endoglycosidase H digestion of HN immunoprecipitated by group I monoclonal antibodies is consistent with the 63,000-molecular-weight polypeptide immunoprecipitated from tunicamycin-treated infected cells. When the HN polypeptide immunoprecipitated by group II monoclonal antibodies was subjected to an identical endoglycosidase H digestion, two polypeptides were produced. This indicates that there are two populations of HN polypeptides recognized by group II monoclonal antibodies that differ in their number of high-mannose oligosaccharide side chains. It is likely that the 64,000-molecular-weight endoglycosidase H digestion product represents deglycosylation of the 74,000-molecular-weight polypeptide, whereas the 69,000-molecular-weight endoglycosidase H digestion product represents the partially deglycosylated 160,000-molecular-weight polypeptide, now in its reduced form. Further posttranslational processing of some of the high-mannose oligosaccharide side chains on the 160,000-molecular-weight polypeptide into the endoglycosidase H-resistant complex side-chains would explain these results.

The above interpretations are further supported by the analysis of infected cells labeled with [^{3}H]mannose and [^{3}H]fucose. Mannose is one of the first carbohydrates added to glycoproteins, whereas fucose is not added until late in posttranslational processing, somewhere in or after the *trans*-Golgi apparatus (19). Group I monoclonal antibodies immunoprecipitated the 76,000-molecular-weight glycopolypeptide from cells labeled with [^{3}H]mannose; however, nothing was immunoprecipitated with these antibodies from cells labeled with [^{3}H]fucose. Group II monoclonal antibodies immunoprecipitated the 74,000- and 160,000-molecular-weight polypeptides from infected cells labeled with [^{3}H]mannose. However, only the 160,000-molecular-weight polypeptide was identified by group II antibodies when infected cells labeled with [^{3}H]fucose were analyzed. These results show that the intermolecular disulfide bond formation that occurs during the maturation of HN occurs before entry into the *trans*-Golgi apparatus, where fucose is added.

The group I monoclonal antibodies clearly recognized an early posttranslational form of the HN protein. Therefore, it was not surprising that they failed to recognize HN on the surface of the infected cell. Group II monoclonal antibodies did recognize HN on the infected-cell surface, which is predominantly the 160,000-molecular-weight, or $(HN)_2$ form. However, some 74,000-molecular-weight HN also migrated to the cell surface, where it was recognized by some, but not all, group II monoclonal antibodies. Only $(HN)_2$ was incorporated into mature virus particles, leaving the presence of the 74,000-molecular-weight HN on the surface of the cell unexplained.

We attempted to visually determine where in the infected cell the conformational change in HN occurred by using group I and group II monoclonal antibodies in an indirect immunofluorescence assay. Both antibody groups produced identical staining patterns, regardless of the fixatives chosen. This suggests that these fixatives denature HN sufficiently to permit group I antibodies to bind wherever HN is localized.

Several interesting aspects of how protein modifications can affect the conformation of a protein are addressed by this study. For instance, only the group I antibodies could immunoprecipitate the 63,000-molecular-weight unglycosylated form of the HN polypeptide. Therefore, formation of the antigenic site recognized by group II antibodies is dependent on posttranslational processing. The addition of oligosaccharide side chains to polypeptides has been postulated as a mechanism for stabilizing specific conformations of polypeptides (7, 11, 13). We show here by immunochemical techniques that this is also true for the mumps virus HN glycoprotein. Proper glycosylation is required for the HN of Newcastle disease virus to be biologically active (17). Maturational studies of Newcastle disease virus glycoproteins have shown that the biologically active form of HN accumulates mainly in the plasma membrane of the cell and that the disulfide-bonded dimer of HN is the predominate form that accumulates (22). The dimer is also the only form of HN present in Newcastle disease virus virions (24). The present studies show a similar maturation pattern for the mumps virus HN glycoprotein.

The increase in electrophoretic mobility between the immature (molecular weight, 76,000) and mature (molecular weight, 74,000) intracellular monomers of the mumps virus HN could be explained by several mechanisms: (i) loss of a portion of the polypeptide by cleavage of a precursor molecule, (ii) trimming and processing of oligosaccharide side chains, or (iii) intramolecular disulfide bond formation. Cleavage of a signal peptide is an inviting possibility, but signal peptide removal appears to be an early event in the translation of membrane-bound proteins, and the molecular weight change observed for the intracellular HN monomers occurs after the molecule is fully translated and at least partially glycosylated. Further, recent evidence suggests that paramyxovirus HN proteins are anchored in the lipid bilayer by an extended signal sequence at their amino termini (2, 9, 21), which would make signal peptide removal incompatible with integration into the lipid bilayer. Processing of the HN glycoprotein of some Newcastle disease virus strains includes conversion of a precursor HN_o to the active form of HN by proteolytic removal of a 9,000-molecular-weight peptide (6). That a similar process exists for mumps virus HN is a possibility that we must investigate further. Deglycosylation could also cause a decrease in the apparent molecular weight of HN. However, this effect should not be augmented by analyzing samples without reduction. Clearly, both types of HN monomers showed a greater difference in heterogeneity and electrophoretic mobility when the two polypeptides were analyzed without disulfide bond reduction. These results support intramolecular disulfide bonding as a plausible explanation for the difference between immature and mature mumps virus HN monomers. In such a model, the newly stabilized intramolecularly bonded conformation of HN would no longer bind group I antibodies but would permit the binding of group II antibodies. This stabilized conformation of the mature HN monomer appears to be a prerequisite for its intermolecular disulfide bond formation to form the oligomers present on the surface of infected cells and in mature mumps virus.

In conclusion, the mumps virus HN protein is translated

and cotranslationally N-link glycosylated into a 76,000-molecular-weight polypeptide. This polypeptide is then stabilized by intramolecular disulfide bonds, which changes its apparent molecular weight to 74,000. These 74,000-molecular-weight monomers are then intermolecularly disulfide bonded into 160,000-molecular-weight dimers. Some of the simple oligosaccharide side chains of the dimers are then processed into complex forms as the molecules move to the surface of the infected cell. These mature 160,000-molecular-weight forms of HN are ultimately incorporated into the virus lipid envelope as virus particles mature at the plasma membrane.

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