JOURNAL OF VIROLOGY, Mar. 1985, p. 761–770
0022-538X/85/030761-10$02.00/0

Vol. 53, No. 3

Structural Analysis of the Varicella-Zoster Virus gp98-gp62 Complex: Posttranslational Addition of *N*-Linked and *O*-Linked Oligosaccharide Moieties

EDUARDO A. MONTALVO, RICHARD T. PARMLEY, AND CHARLES GROSE†*

Department of Pediatrics and Department of Microbiology, The University of Texas Health Science Center at San Antonio, San Antonio, Texas 78284

Received 13 August 1984/Accepted 16 November 1984

Varicella-zoster virus specifies the formation of several glycoproteins, including the preponderant gp98-gp62 glycoprotein complex in the outer membranes of virus-infected cells. These viral glycoproteins are recognized and precipitated by a previously described monoclonal antibody designated monoclone 3B3. When an immunoblot analysis was performed, only gp98 was reactive with monoclone 3B3 antibody; likewise, titration in the presence of increased concentrations of sodium dodecyl sulfate during antigen-antibody incubations caused selective precipitation of gp98 but not gp62. Further structural analyses of gp98 were performed by using the glycosidases endo-β-*N*-acetylglucosaminidase H (endoglycosidase H) and neuraminidase and two inhibitors of glycosylation (tunicamycin and monensin). In addition to gp98, antibody 3B3 reacted with several intermediate products, including gp90, gp88, gp81, and a nonglycosylated polypeptide, p73. Since gp98 was completely resistant to digestion with endoglycosidase H, it contained only complex carbohydrate moieties; conversely, gp81 contained mainly high-mannose residues. Polypeptide p73 was immunodetected in the presence of tunicamycin and designated as a nascent recipient of *N*-linked sugars, whereas gp88 was considered to contain *O*-linked oligosaccharides because its synthesis was not affected by tunicamycin. The ionophore monensin inhibited production of mature gp98, but other intermediate forms, including gp90, were detected. Since the latter product was similar in molecular weight to the desialated form of gp98, one effect of monensin treatment of varicella-zoster virus-infected cells was to block the addition of *N*-acetylneuraminic acid. Monensin also blocked insertion of gp98 into the plasma membrane and, as determined by electron microscopy, inhibited envelopment of the nucleocapsid and its transport within the cytoplasm. On the basis of this study, we reached the following conclusions: (i) the primary antibody 3B3-binding epitope is located on gp98, (ii) gp98 is a mature product of viral glycoprotein processing, (iii) gp98 contains both *N*-linked and *O*-linked oligosaccharide side chains, (iv) gp90 is the desialated penultimate form of gp98, (v) gp88 is an *O*-linked intermediate of gp98, (vi) gp81 is the high-mannose intermediate of gp98, and (vii) p73 is the unglycosylated precursor of gp98.

The genome of varicella-zoster virus (VZV) codes for the formation of several glycoproteins which are synthesized in abundance within infected cells and are also incorporated into the envelopes of the virions. On the basis of their relative mobilities during sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) under reducing conditions, these glycoproteins have been designated by their apparent molecular weights as follows: gp118, gp98, gp88, gp62, and gp45 (10). We have also produced panels of murine monoclonal antibodies which precipitate these glycosylated proteins, identify previously unrecognized viral glycoproteins, and define some of their biological properties (11, 12). For example, we have observed that an anti-gp118 monoclonal antibody identifies an epitope which elicits a complement-independent neutralizing antibody response (11). On the basis of its chromatographic properties, this glycoprotein appears to contain mainly *N*-linked, complex and hybrid carbohydrate moieties (9). Since gp118 is found in the enveloped virions, it is presumably a mature form of the glycoprotein. In a subsequent hybridoma cell fusion, we produced two monoclonal antibodies which precipitate a sixth glycoprotein, gp140, a disulfide-linked mature product which is cleaved to gp66 under reducing conditions (12).

Interestingly, when we analyzed the protein specificities of four of the original monoclonal antibodies, we observed that they precipitated more than one fucosylated protein (11). In particular, monoclonal antibody 3B3 reacted with both gp98 and gp62 under our standard conditions of solubilization and immunoprecipitation. The same glycoproteins also were the preponderant species inserted into the plasma membranes of VZV-infected cells, as well as major components of VZV-induced syncytia (38). Based on prior investigations of the individual VZV glycoproteins during primary VZV infection (chicken pox) and reactivation (zoster) in humans, these two VZV-specified cell surface glycoproteins undoubtedly participate in the pathogenesis of VZV infection and also mediate important host immune responses to virus infection (39). Therefore, in this study we further characterized the biochemistry and immunobiochemistry of the VZV-specific gp98-gp62 glycoprotein complex. We identified nonglycosylated precursors, as well as intermediate forms of the mature glycoprotein which occurred during oligosaccharide processing. For these analyses, we used two inhibitors of glycosylation (tunicamycin and monensin) and two glycosidases (endo-β-*N*-acetylglycosaminidase H [Endo H] and neuraminidase). Finally, we correlated perturbations

* Corresponding author.
† Present address: Department of Pediatrics, Division of Infectious Diseases, University of Iowa Hospitals, Iowa City, IA 52242.

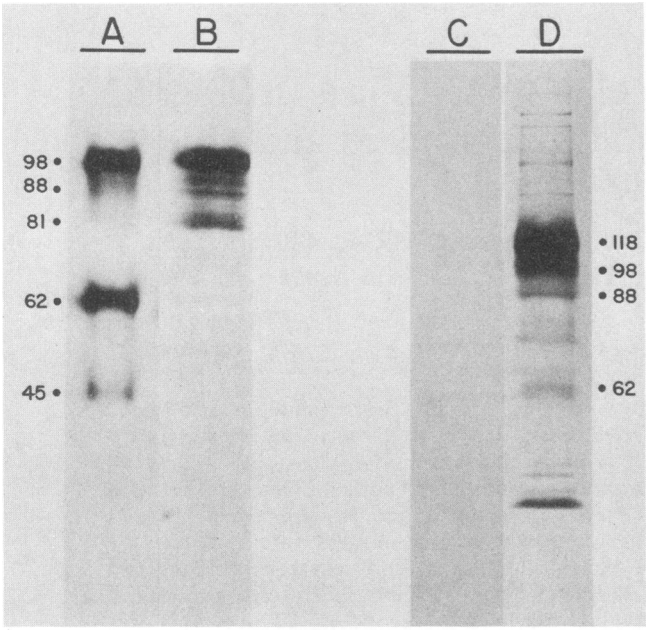


FIG. 1. Immunoprecipitation profile compared with immunoblotting profile obtained with monoclonal antibody 3B3. [^{3}H]fucose-labeled VZV-infected cell lysates were incubated with monoclonal antibody 3B3, and the immunoprecipitates were collected on protein A-Sepharose beads. Eluted proteins were fractionated by electrophoresis in a 10% SDS–polyacrylamide gel (lane A). Alternatively, unlabeled VZV-infected cell lysates were subjected to SDS-PAGE, and the fractionated proteins were subsequently transferred to nitrocellulose sheets. Nitrocellulose replicas were incubated with monoclonal antibody 3B3 for 1 h. Bound antibody was detected indirectly with peroxidase-labeled goat anti-mouse antibody. Color development was effected by the addition of 4-chloro-1-naphthol substrate, followed immediately by 30% H_2O_2 (lane B). In a separate control experiment, uninfected cells (lane C) or VZV-infected cells (lane D) were subjected to electrophoresis and transferred to nitrocellulose sheets, as described above. The sheets were then incubated with guinea pig polyclonal VZV antiserum for 1 h, washed, and incubated with peroxidase-labeled anti-guinea pig antibody. Immunoreactive VZV antigens were detected as previously described (6, 37). Nitrocellulose replicas of uninfected cells also were incubated with monoclonal antibody 3B3 and were as nonreactive as the immunoblot shown in lane C. The molecular weights ($\times 10^3$) of major VZV glycoproteins, as previously designated by workers in our laboratory (10, 11, 12, 13), are indicated on the left and on the right in this and subsequent figures.

in glycosylation with lack of envelopment and loss of infectivity.

MATERIALS AND METHODS

Cells and virus. The Mewo strain of human melanoma cells (HMC) was grown at 36°C in Eagle minimal essential medium supplemented with 0.002 M glutamine, 1% nonessential amino acids, 100 U of penicillin per ml, 100 μg of streptomycin per ml, and 10% fetal bovine serum. The virus stock used for all experiments was strain VZV-32 (10).

Infection of cells and intrinsic radiolabeling. HMC monolayers (75 cm^2) that were 1 day old were inoculated with one-fourth equivalent of trypsin-dispersed VZV-infected cells and incubated at 32°C. At 18 h postinfection, the culture medium was replaced with minimal essential medium supplemented with either 300 μCi of [5,6-^{3}H]fucose (specific activity, 56 Ci/mmol), 125 μCi of L-[*U*-^{14}C]amino acids (specific activity, >50 mCi/mg · atom of carbon), or 250 μCi of D-[*U*-^{14}C]glucosamine hydrochloride (specific activity, 200 mCi/mmol). The cultures were incubated for an additional 24 h at 32°C and harvested with a rubber policeman. Cell lysates were prepared as previously described (10, 12).

Immunoprecipitation. The techniques used for radioimmune precipitation with staphylococcal protein A bound to Sepharose CL-4B beads (Pharmacia Fine Chemicals, Inc.) have been described previously (11, 12).

Digestion with Endo H and neuraminidase. The methods used for digestion with Endo H (EC 3.2.1.96; Miles Laboratories, Inc.) have been described previously (40). Briefly, glycoproteins immunoprecipitated with monoclonal antibody were washed twice with 0.5 M lithium chloride–0.1 M Tris (pH 8.8). The glycoproteins were eluted from Sepharose-protein A beads by incubating the samples in 0.8% SDS for 20 min at 37°C and then boiling for 2 min. The Sepharose beads were removed by low-speed centrifugation. Sodium citrate buffer (pH 5.5) was added to give final concentrations of 0.05 M buffer and 0.2% SDS. Endo H (60 μl of a 0.1-U/ml solution) or water (60 μl) was added to each sample. After incubation for 6 h at 37°C, the proteins were precipitated with cold acetone, washed three times with cold ethanol, and suspended in 50 μl of SDS sample buffer. The experimental conditions used for the neuraminidase experiments were the same as those described above, except that sodium citrate buffer was replaced by 0.05 M sodium acetate buffer (pH 5.3) containing 0.1 M calcium chloride (28). Neuraminidase from *Clostridium perfringens* (EC 3.2.1.18; Sigma Chemical Co.) was added at a final concentration of 15 mU/ml.

Preparation of antigen for immunoblotting. HMC monolayers (25 cm^2) that were 1 day old were inoculated with one-fourth equivalent of trypsin-dispersed VZV-infected cells and incubated at 32°C. At 34 h postinfection, the cells were dislodged with a rubber policeman and suspended in 1 ml of 0.15 M NaCl–0.01 M Tris (pH 7.4). The cell suspension was sonically disrupted for 2 min, and the cellular debris was removed by centrifugation for 5 min at 1,500 rpm and 4°C. Antigen preparations were stored at −70°C. For experiments with monensin and tunicamycin (both obtained from Calbiochem-Behring), the culture medium was replaced by medium supplemented with 1 μM monensin or 2.5 μg of tunicamycin per ml, as described below.

SDS-PAGE. Polyacrylamide slab gels containing 0.1% (vol/vol) SDS were prepared from acrylamide cross-linked with *N*,*N*′-methylene bisacrylamide at a ratio of 77:1. Electrophoresis was carried out by standard procedures in a Tris-glycine-SDS buffer system (pH 8.1) at ambient temperature (10). The slab gels were suffused with 2,5-diphenyloxazole for fluorographic enhancement (5) before drying and exposure to radiographic film at −70°C. For immunoblot analysis, proteins were transferred to nitrocellulose paper as described below.

Immunoblotting techniques. Transfer of proteins from slab gels to nitrocellulose paper was performed by the method of Towbin et al. (37), as modified by Burnette (6). Electroelution was carried out at 150 mA for 14 h in 25 mM Tris–192 mM glycine containing 20% (vol/vol) methanol at pH 8.3 in a Hoefer apparatus. Immediately after transfer, the nitrocellulose sheets were immersed for 3 h at 37°C in NET (0.15 M NaCl, 0.05 M tetrasodium EDTA, 0.05 M Tris, pH 7.4) supplemented with 0.25% gelatin, 0.05% Nonidet P-40 (NP-40), and 5% bovine serum albumin. The sheets were incubated with monoclonal antibody (diluted in NET containing gelatin, NP-40, and bovine serum albumin) for 1 h at

ambient temperature. After antigen-antibody binding, the sheets were washed six times with NET containing gelatin and NP-40 on a shaker. Bound antibody was detected during a 1-h incubation at ambient temperature with an indicator conjugate (horseradish peroxidase-labeled goat anti-mouse antibody [1:100 dilution in NET containing gelatin, NP-40, and bovine serum albumin]). The sheets were washed once with NET containing gelatin and NP-40 and three times with 0.15 M NaCl–0.05 M Tris (pH 7.4). The sheets were finally submerged in 50 ml of cold 0.15 M NaCl–0.05 M Tris (pH 7.4) to which we added 10 ml of substrate solution (3 mg of 4-chloro-1-naphthol per ml of methanol), followed by 50 μl of 30% H_2O_2. After color development, the sheets were washed for 5 min with 0.15 M NaCl–0.05 M Tris (pH 7.4).

RESULTS

Comparison of immunoprecipitation and immunoblotting. Monoclone 3B3 secretes an immunoglobulin G2a which elicits an intense immunostaining pattern within the cytoplasm and on the plasma membranes of VZV-infected cells (38). This antibody also binds avidly to staphylococcal protein A and thereby exhibits superior precipitating capacity (11). Therefore, we selected antibody 3B3 as a probe to further investigate the gp98-gp62 complex. We reported previously that two proteins (gp98 and gp62) are consistently detected in monoclone 3B3 immunoprecipitates of [^{3}H]fucose-labeled detergent-solubilized VZV-infected cell lysates (11). Occasionally, we observed two other proteins, gp88 and gp45, which were more easily detected after longer exposures of the fluorogram (Fig. 1, lane A).

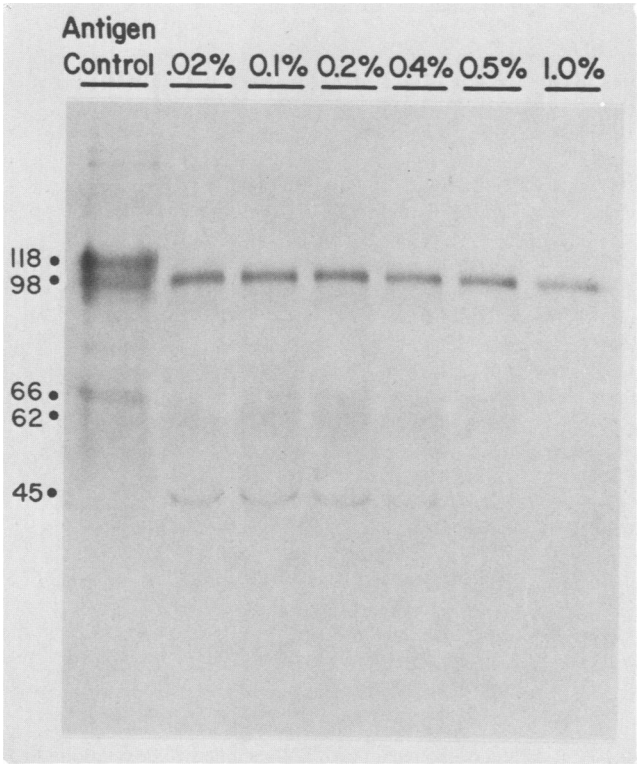


FIG. 2. Effect of increasing SDS concentration in the antigen-antibody reaction mixture. [^{3}H]fucose-labeled VZV-infected cell lysates were solubilized and incubated with antibody 3B3 in the presence of 0.02, 0.1, 0.2, 0.4, 0.5, and 1.0% SDS, and antigen-antibody complexes were precipitated with protein A-Sepharose beads. All buffers used during immunoprecipitation contained the same SDS concentrations that were present in the respective antigen-antibody reaction mixtures. Bound antigen was eluted and subjected to SDS-PAGE in a 10% acrylamide slab gel.

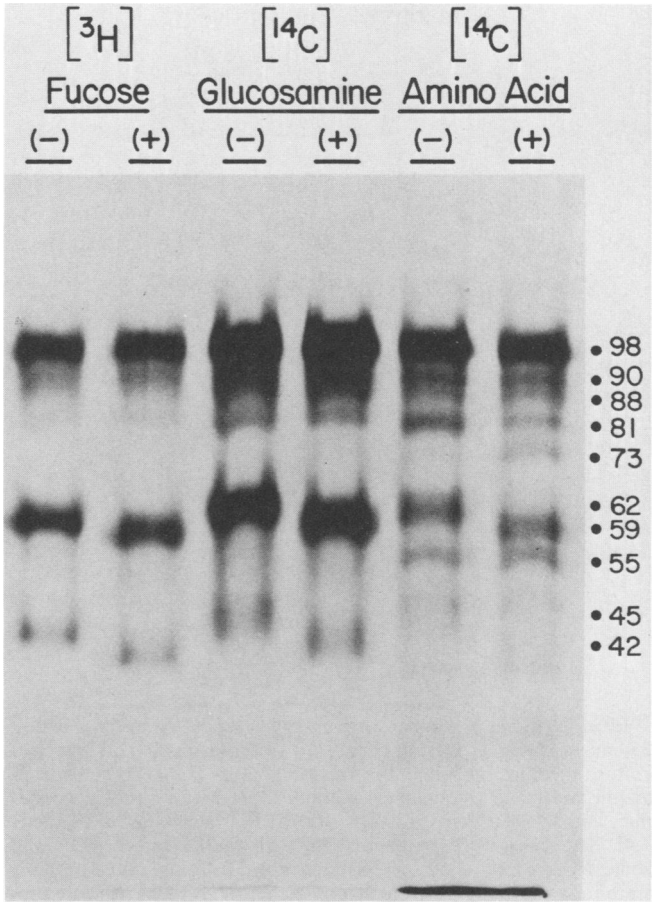


FIG. 3. Endo H digestion of polypeptides immunoprecipitated with monoclonal antibody 3B3. VZV-infected cell lysates labeled with [^{3}H]fucose, [^{14}C]glucosamine, or ^{14}C-amino acids were immunoprecipitated with antibody 3B3. The precipitated polypeptides were eluted, suspended in 0.05 M sodium citrate buffer (pH 5.5), and incubated in the absence (−) or presence (+) of enzyme (60 μl of a 0.1-U/ml solution). Proteins were then precipitated with cold acetone and subjected to SDS-PAGE in a 10% acrylamide slab gel.

To determine whether antibody could bind denatured glycopeptides, an immunoblot analysis was performed. SDS-PAGE profiles of VZV-infected cell lysates were transferred onto nitrocellulose sheets, and the nitrocellulose replicas were incubated with antibody 3B3. A second incubation with peroxidase-labeled goat anti-mouse antibody led to subsequent detection of antibody 3B3-specific antigens (Fig. 1, lane B). These included only gp98 and three slightly faster-migrating species, gp90, gp88, and gp81. Neither gp62 nor gp45 was detected in the nitrocellulose transfers of SDS-PAGE-separated proteins. The results were the same under both reducing and nonreducing conditions.

Effect of SDS concentration in the antigen-antibody reaction mixture. As described above, immunoblotting revealed the failure of antibody 3B3 to bind denatured gp62-gp45. We also examined the effect of increasing concentrations of SDS on antigen-antibody binding. Immunoprecipitation was carried out as described above but with increasing SDS concentrations. As Fig. 2 shows, the amount of gp62-gp45

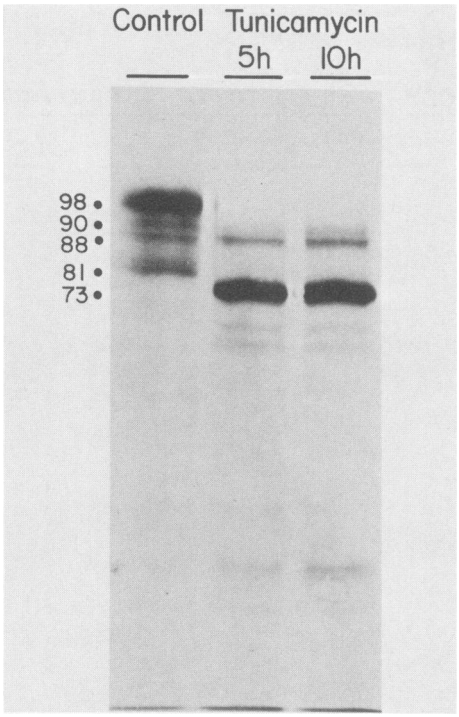


FIG. 4. Effect of tunicamycin on gp98 processing. VZV-infected 25-cm^2 monolayers were incubated with tunicamycin (2.5 μg/ml of culture medium) at 5 and 10 h postinfection. Tunicamycin-treated and untreated cell monolayers were dislodged with a rubber policeman and washed three times with 0.15 M NaCl–0.01 M Tris (pH 7.4). The cells were suspended in 1 ml of the same buffer and sonically disrupted for 2 min. Electrophoretically separated proteins were transferred to nitrocellulose paper and then subjected to immunoblot analysis with antibody 3B3 as described in the legend to Fig. 1. The products were fractionated in a 10% acrylamide slab gel.

precipitated by antibody 3B3 depended upon the amount of SDS present in the reaction mixture. At an SDS concentration of 1%, only gp98 was bound by antibody. Using this procedure to test our other monoclonal antibodies which precipitated the gp98-gp62 complex, we were able to selectively precipitate either gp98 or gp62 at higher SDS concentrations. These results again suggested that the true epitope recognized by antibody 3B3 resided on gp98 and not on either of the two lower-molecular-weight species, gp62 and gp45.

Digestion with Endo H. Antibody 3B3 immunoprecipitates of isotopically labeled VZV-infected cells were examined for their susceptibility to digestion with Endo H. Endo H predominantly cleaves between the two proximal *N*-acetylglucosamine residues of high-mannose oligosaccharides. Complex oligosaccharides are resistant to Endo H, whereas intermediate oligosaccharides show intermediate sensitivity (27, 33, 34). VZV-infected cells labeled with [^{3}H]fucose, ^{14}C-amino acids, or [^{14}C]glucosamine were immunoprecipitated with antibody 3B3, washed, and suspended in citrate buffer. The precipitated peptides were incubated in the presence of either Endo H or an equivalent amount of water, after which the proteins were precipitated with cold acetone and subjected to electrophoretic separation.

Because [^{3}H]fucose is incorporated late in glycoprotein processing, we examined the Endo H sensitivity of fucosylated late intermediates or final products. Any detectable Endo H-sensitive oligosaccharides in [^{3}H]fucose-labeled samples presumably would reflect the presence of high-mannose chains in late intermediates or final products. As described above, antibody 3B3 precipitated at least four [^{3}H]fucose-labeled glycopeptides (gp98, gp88, gp62, and gp45). Endo H treatment of antibody 3B3 immunoprecipitates labeled with [^{3}H]fucose caused an increase in the relative migration of gp62 and gp45, whereas the relative migration of gp98 and gp88 remained the same (Fig. 3). The altered migration of both gp62 and gp45 corresponded to a decrease in M_r of 3,000. Based on the assumption that the molecular weight of a high-mannose chain is 1,400 to 1,800 (7, 22), gp62 and gp45 each contained two high-mannose oligosaccharides.

The Endo H sensitivity of [^{14}C]glucosamine-labeled precipitates was also examined. This amino sugar is incorporated into glycoproteins both before and after acquisition of Endo H resistance. An examination of samples incubated in the absence of Endo H allowed detection of another gp98 precursor, gp81. With the exception of less label incorporation by gp81 in Endo H-treated samples, no previously undetected proteins were observed.

To look for unglycosylated precursors to the gp98-gp62 complex, VZV-infected cultures were isotopically labeled with ^{14}C-amino acids and incubated in the absence and

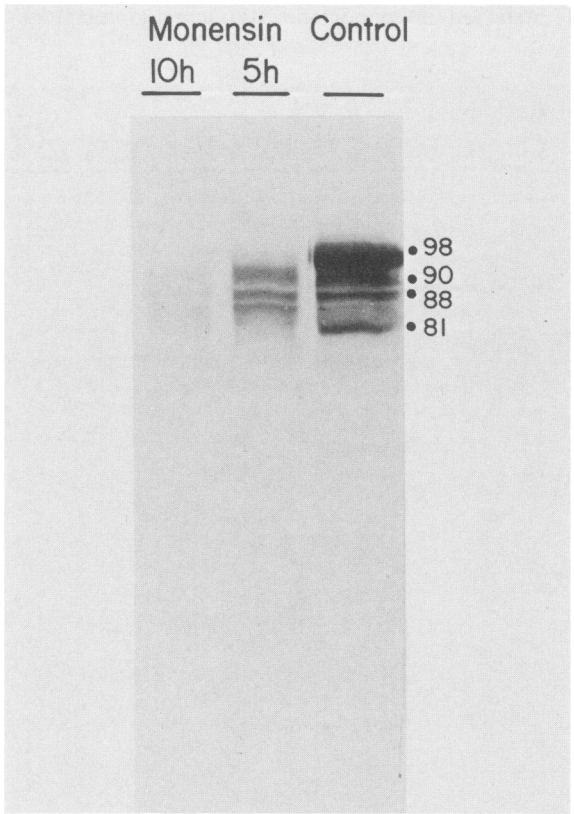


FIG. 5. Effect of monensin on VZV-specified glycosylation. At 5 and 10 h postinfection, monensin (1 μM) was added to the culture medium. At 34 h postinfection, monensin-treated and untreated control infected cell monolayers were dislodged with a rubber policeman, washed three times with 0.15 M NaCl–0.01 M Tris (pH 7.4), sonically disrupted, and subjected to SDS-PAGE on 10% acrylamide slab gels. The separated proteins were transferred to nitrocellulose sheets, and the nitrocellulose replicas were incubated with antibody 3B3 for 1 h at ambient temperature. Reactive proteins were detected by immunoblot analysis.

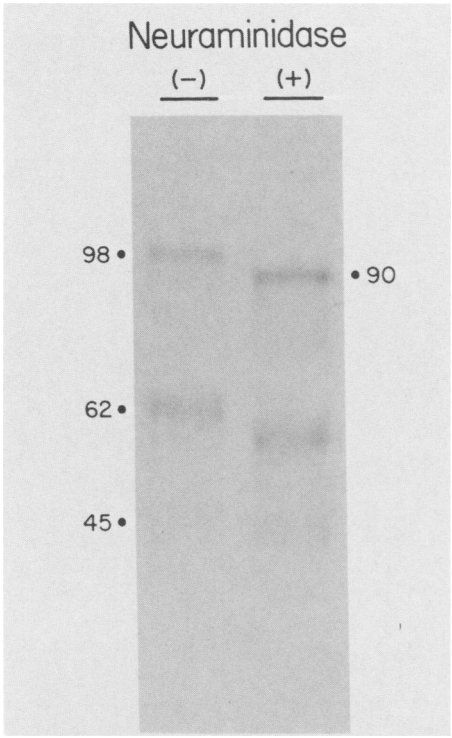


FIG. 6. Neuraminidase treatment of glycopolypeptides immunoprecipitated by monoclonal antibody 3B3. [^{3}H]fucose-labeled VZV-specific glycoproteins were immunoprecipitated with antibody 3B3. Eluted glycopeptides were suspended in 0.05 M sodium acetate buffer (pH 5.5) and incubated for 6 h in the absence (−) or presence (+) of neuraminidase (15 mU/ml). Digested products were fractionated in a 10% polyacrylamide gel.

presence of Endo H. Several observations were made after the fractionated radioimmune precipitates were examined (Fig. 3). (i) What previously appeared as one glycoprotein in fucose- and glucosamine-labeled samples (gp88) was actually two closely migrating species, gp88 and gp90. (ii) gp81 was sensitive to Endo H, although complete removal of high-mannose chains from gp81 did not occur under our experimental conditions; the polypeptide generated from partial removal of high-mannose chains had an M_r of 73,000. (iii) Another faster-migrating species, designated p55, was observed in both Endo H-treated and untreated samples. (iv) A band that migrated slightly faster than gp42 was observed in both treated and untreated controls. Since the p55 band and the band that migrated slightly faster than gp42 were only present in ^{14}C-amino acid-labeled samples, these bands most likely represented the unglycosylated analogs of gp62 and gp45, respectively.

These results revealed three Endo H-sensitive glycoproteins in antibody 3B3 immunoprecipitates. Glycoprotein gp81 was reduced to p73, which was seen only in amino acid-labeled samples. Based on the molecular weight assumption mentioned above, gp81 was generated by the addition of four or five high-mannose chains to p73. In contrast, the Endo H-cleaved products of gp62 and gp45 were observed in fucose-labeled extracts, suggesting the presence of other oligosaccharide constituents which are not of the high-mannose type.

Effect of tunicamycin. Tunicamycin is an antibiotic which inhibits the formation of lipid-linked *N*-acetylglucosamine compounds. In the presence of this antibiotic, the formation of all *N*-linked oligosaccharides is blocked (20, 36). To test whether p73 was the unglycosylated precursor of gp98, tunicamycin was added to VZV-infected HMC monolayers at 5 and 10 h postinfection. The monolayers were harvested 24 h later (i.e., 34 h postinfection), and the cell lysates were subjected to SDS-PAGE. After transfer to nitrocellulose, the VZV-specific polypeptides were immunodetected with antibody 3B3 and a peroxidase-labeled second antibody. As Fig. 4 shows, the addition of tunicamycin to VZV-infected cell monolayers completely blocked the synthesis of gp98. A prominent protein was recognized by antibody 3B3 at an M_r of 73,000. In addition, two faster-migrating polypeptides, which were most likely degradation products, were ob-

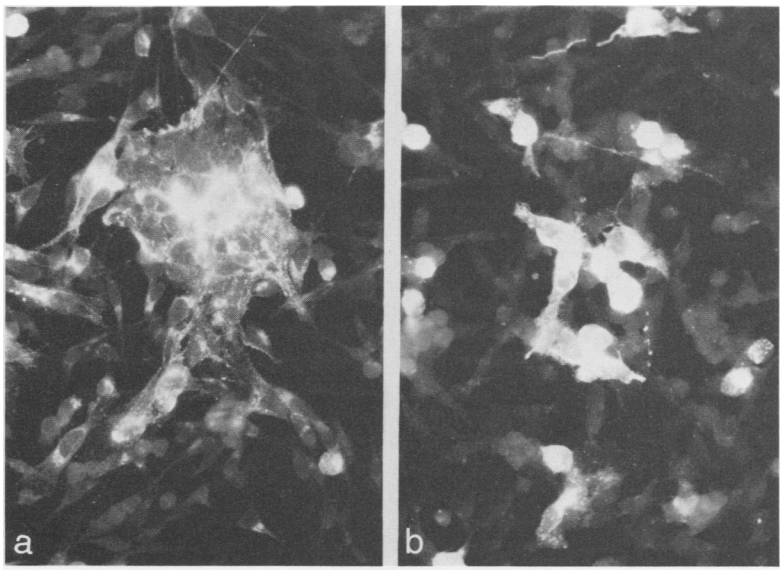
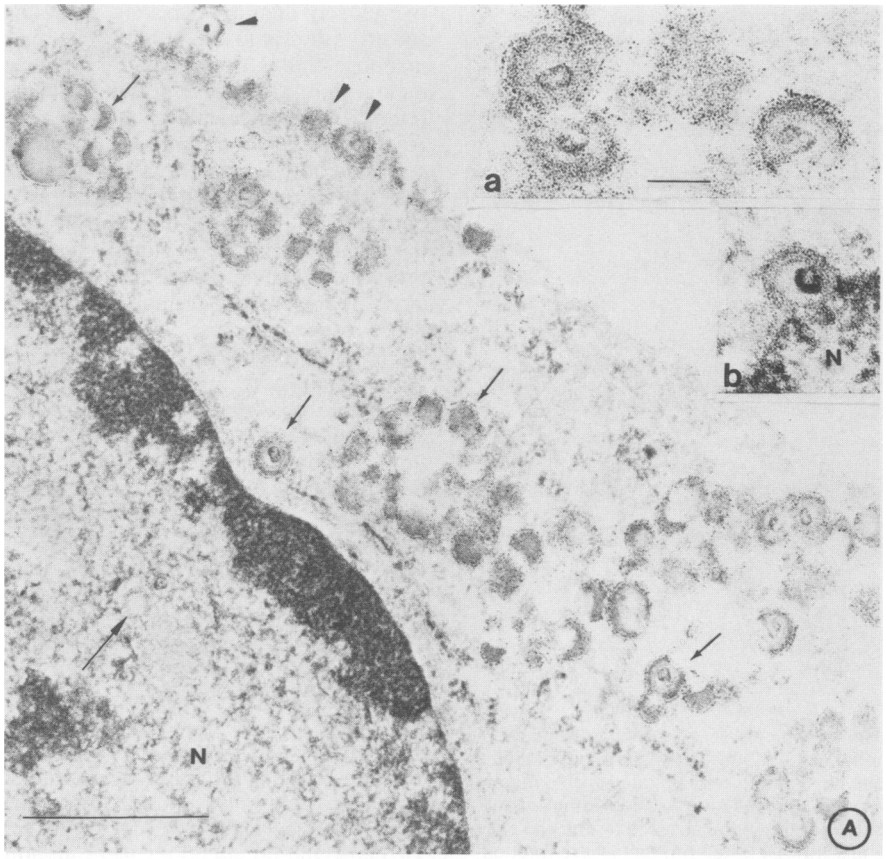
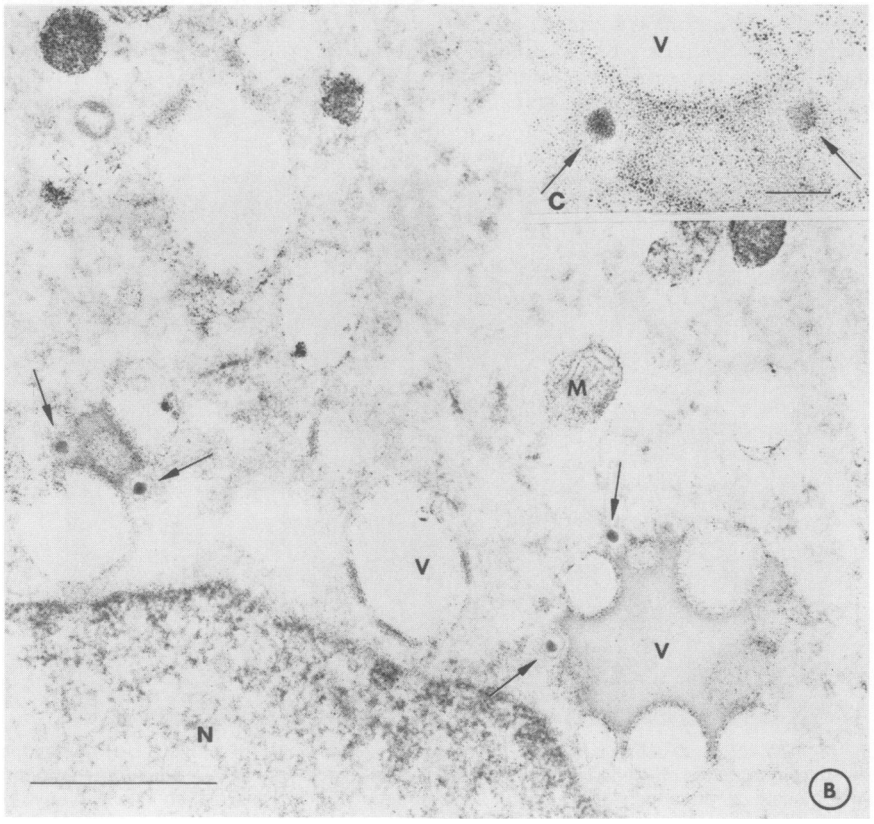


FIG. 7. Immunostaining of VZV-infected cells grown in the presence and absence of monensin. Microscope slides carrying fixed VZV-infected monolayers were rehydrated in phosphate-buffered saline and incubated sequentially with diluted monoclonal antibody 3B3 and goat anti-mouse fluorescein conjugate, as previously described (38). Examination by light microscopy revealed an accumulation of gp98-gp62 reactivity at the juxtanuclear areas in monensin-treated cells (b), whereas untreated VZV-infected cultures (a) exhibited fluorescence throughout the cytoplasm and outer cell membranes of the syncytia.


a
b
N
N
A


V
C
M
V
V
N
B

served in tunicamycin-treated cultures. Similar observations have been made by other investigators, who noted that unglycosylated analogs of some glycoproteins are more susceptible to degradation (20, 25, 26). A glycoprotein with an M_r of 88,000 was also synthesized in the presence of tunicamycin. Since the addition of *O*-linked oligosaccharides to nascent polypeptides is not blocked by tunicamycin (15), gp88 most likely contains *O*-linked oligosaccharides. We also performed indirect immunofluorescence studies of VZV-infected cell monolayers treated with tunicamycin. As reported in other virus-cell systems (20), VZV-specified glycoproteins were not detected at the cell surface in tunicamycin-treated cultures (data not shown).

Effect of monensin. Monensin, a monovalent ionophore, inhibits the transport of glycoproteins specified by vesicular stomatitis virus, Sindbis virus, and herpes simplex virus (2, 17, 18). In addition, this ionophore blocks the processing of *N*-linked oligosaccharides and the addition of *O*-linked oligosaccharides to herpes simplex virus type 1 glycoproteins (19). To examine the effects of monensin on VZV glycoprotein synthesis, monensin was added to VZV-infected HMC monolayers at 5 and 10 h postinfection. Cell lysates were prepared for SDS-PAGE and immunoblotting as described above for tunicamycin-treated cultures. The samples treated with monensin at 5 and 10 h postinfection revealed identical profiles (Fig. 5). Monensin treatment blocked the synthesis of gp98, whereas gp90 accumulated in the ionophore-treated cells. Besides gp90, two additional proteins, gp88 and an unexplained band at an M_r of 84,000, were observed in treated cells.

Digestion with neuraminidase. Sialic acid molecules are often found as the terminal sugars in *N*-linked oligosaccharides and are added, together with fucose, galactose, and glucosamine, during the final stages of processing (16). Similarly, sialic acid signals termination of *O*-linked oligosaccharide processing (3, 4). To test whether sialic acid could account for observed differences between monensin-treated and untreated samples, we added neuraminidase to antibody 3B3 immunoprecipitates of isotopically labeled VZV-infected cells. After 6 h of incubation, the samples were washed, precipitated with cold acetone, and examined by SDS-PAGE. All three glycoproteins (gp98, gp62, and gp45) were sensitive to neuraminidase (Fig. 6). The large increase in the mobility of enzyme-treated polypeptides compared with untreated controls suggested that the glycopeptides were heavily sialated. Removal of *N*-acetylneuraminic acid residues from gp98 generated a protein with a M_r of 90,000. Therefore, one effect of monensin on VZV glycoprotein synthesis was to block sialation of glycoproteins. Furthermore, gp62 and gp45, which were previously shown to be Endo H sensitive, contained fully processed oligosaccharides and were therefore not precursors in oligosaccharide processing.

Light and electron microscopic analyses of monensin-treated cultures. Examination of VZV-infected cells by indirect immunofluorescence with antibody 3B3 revealed reactive sites throughout the cytoplasm and outer cell membranes of the syncytia; in the presence of monensin, there was an accumulation of gp98-gp62 at juxtanuclear areas, with virtually no detectable antigen in the cytoplasm or infected cell membranes (Fig. 7). The same cultures were prepared for electron microscopic visualization by the periodate-thiocarbohydrazide-silver proteinate method, which stains glycol-containing complex carbohydrates (8). In the untreated infected culture, nucleocapsids left the nucleus but did not acquire periodate-reactive envelopes as they passed through the outer nuclear membrane; rather, the nucleocapsids were enveloped as they entered intracytoplasmic vacuoles, which have been described previously (1, 14). By using periodate-thiocarbohydrazide-silver proteinate staining, we observed reactive glycoproteins along the inner membranes of the vacuoles and throughout the envelopes of the virions within each vacuole (Fig. 8A). Reactive glycoconjugates also were present in the outer cell membranes of the infected cells. In marked contrast, nucleocapsids in the monensin-treated culture entered the cytoplasm but subsequently failed to enter the vacuoles and acquire periodate-reactive envelopes (Fig. 8B). Thus, monensin treatment of VZV-infected cultures blocked the envelopment of nucleocapsids within the cytoplasm and also inhibited further transit of viral particles through the cytoplasm to the outer cell membrane.

This electron microscopic localization of periodate-reactive glycoconjugates corroborated and expanded the topographic differences delineated by immunostaining (Fig. 7). Furthermore, these studies demonstrated that a major portion of the VZV virion envelope was acquired within the cytoplasm rather than from the outer nuclear membrane. We have not yet investigated whether sites of virion assembly may differ in substrates other than HMC; however, similar large intracytoplasmic vacuoles filled with virions have been observed in VZV-infected human amnion cells (1). When VZV-infected HMC cultures incubated in the presence of monensin were harvested and sonically disrupted, there was a decrease in infectivity of more than 1,000-fold (<10 PFU/culture) compared with untreated companion cultures. Thus, the ionophore-induced perturbations in virus-specified glycosylation virtually eliminated infectivity.

DISCUSSION

VZV specifies the formation of at least six glycoproteins in infected cell cultures; these glycoproteins, which have been designated gp118, gp98, gp88, gp66, gp62, and gp45, are also present in the enveloped virions. By analyzing several panels of VZV-specific monoclonal antibodies, we discov-

FIG. 8. Electron microscopic visualization of periodate-reactive glycoproteins in VZV-infected cells. VZV-infected cultures grown under normal conditions in the absence of monensin (A) and companion cultures incubated in the presence of 1 μM monensin (B) were harvested, and the monolayers were prepared for cytochemical localization of periodate-reactive complex carbohydrates exactly as described by Fittschen et al. (8). (A) VZV nucleocapsids (long arrow) within the nucleus (N), clusters of enveloped particles (short arrows) within cytoplasmic vacuoles, and virions (arrowheads) adherent to the outer cell membrane. Individual viral particles are indicated by arrows. Periodate-reactive complex carbohydrates within the envelope of a virion were identified by the presence of darkly staining silver granules. This reactivity is clearly evident in inset a, which illustrates three mature enveloped particles. Inset b shows a relatively unreactive viral particle emerging from the nucleus. Lower bar = 0.5 μm. Upper bar (inset a) = 0.1 μm. (B) Effect of monensin on the assembly of VZV virions. A few viral particles (arrows) are evident within the nucleus (N) and cytoplasm. However, the viral particles have not entered the cytoplasmic vacuoles (V), nor do they possess periodate-reactive envelopes. This lack of complex carbohydrates within the viral envelope was clearly evident under higher magnification (inset c). M, Melanosome. Lower bar = 0.5 μm. Upper bar (inset c) = 0.1 μm.

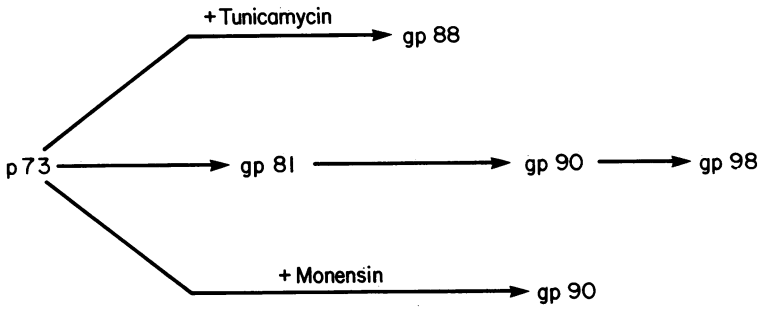


FIG. 9. Schematic representation of the processing of gp98. The unglycosylated analog of gp98 is p73. The addition of high-mannose oligosaccharides to the peptide backbone results in the generation of gp81, the high-mannose intermediate form. After processing of the *N*-linked sugars and the addition of *O*-linked residues, gp90 is formed. The final addition of sialic acid moieties to gp90 results in the mature product, gp98. In the presence of tunicamycin, a gp88 form is synthesized, which represents a product lacking *N*-linked oligosaccharide chains. The ionophore monensin causes a block in the addition of sialic acid to the oligosaccharides.

ered that one antibody (clone 9C6) precipitated gp118, which we have subsequently purified by serial affinity chromatography and thereby characterized as an asparagine-linked fucosylated complex carbohydrate (9). Two monoclonal antibodies precipitated disulfide-linked glycoprotein gp140, which was cleaved mainly into the reduced product gp66 (12). Four monoclonal antibodies in our initial panel reacted with the gp98-gp62 glycoprotein complex. The same two glycoproteins also were prominent components of VZV-infected cell membranes. In this study we extended our previous observations about this glycoprotein complex.

Using antibody 3B3, we further defined the location of the binding site to gp98 and not to gp62 or gp45. The evidence for this specificity is twofold; (i) only gp98 and its precursor forms were detected by immunoblotting after transfer of the proteins to nitrocellulose paper, and (ii) increasing concentrations of SDS during immunoprecipitation inhibited reaction with gp62 and gp45 but not gp98. In the presence of 1% SDS in both the initial antigen preparation and all buffers, antibody 3B3 precipitated only gp98. Presumably, therefore, the epitope is defined by a primary sequence of amino acids which is not altered by loss of tertiary conformation of the glycoprotein after either SDS treatment or immunoblotting. Our results are consistent with the observations of Okuno et al. (24), who noted that five of their monoclonal antibodies precipitated a glycoprotein complex with 94,000- and 55,000-dalton constituents. By using pulse-chase experiments these authors proved that the 94,000-, 83,000-, and 75,000-dalton glycopeptides were distinct from the 55,000- and 49,000-dalton species. Although there are small differences in M_r estimates, we believe that the glycoprotein complex described above is the same as that precipitated by antibody 3B3. The nature of the tight association of gp62 and gp45 to gp98 remains to be clarified. Preliminary data from our laboratory suggest that these glycoproteins form a protein complex through ionic interactions. Noncovalent associations of membrane glycoproteins have been described for the major histocompatibility complex molecules (23), as well as for platelet membrane glycoproteins (21).

After determining that gp98 and gp62 were different glycoproteins, we performed structural analyses of their intermediate and mature forms (Fig. 9). The formation of *N*-acetylglucosamine intermediates is the first step in *N*-linked glycosylation. The high-mannose oligosaccharides are next transferred en bloc from the dolichol carrier to the acceptor asparagine residue on the protein (32). Since this step is inhibited by tunicamycin, we added tunicamycin to VZV-infected cells and identified an unglycosylated polypeptide, p73, with our monoclonal antibody probe. Furthermore, after intrinsic radiolabeling with [^{3}H]fucose, [^{14}C]glucosamine, and ^{14}C-amino acids, p73 was the last nonglycosylated protein identified before the stepwise addition of sugar moieties. Therefore, we conclude that p73 is the nonglycosylated precursor of gp98.

High-mannose intermediate forms were detected after the addition of the glycosidase Endo H to glycoproteins immunoprecipitated with monoclonal antibody 3B3. After digestion with Endo H, the mobility of gp81 was reduced to the mobility of a molecule with an M_r of 73,000, a change consistent with the results observed after tunicamycin treatment. Therefore, gp81 is the high-mannose intermediate precursor of gp98. Because gp98 was completely insensitive to Endo H digestion, all high-mannose chains in the mature glycoprotein underwent further processing. We also observed that both gp62 and gp45 contained high-mannose chains because their M_r values were reduced after digestion with the glycosidase.

Our observations also suggested that gp98 contained oligosaccharides of the *O*-linked type in addition to complex *N*-linked side chains. In the presence of tunicamycin a precursor glycoprotein, gp88, was synthesized. It is possible that the putative *O*-linked gp88 is not identical to the 88,000-dalton species previously observed in infected cells but simply comigrates under SDS-PAGE conditions. However, both gp98 and gp88 are found in purified virions (13, 24), and pulse-chase experiments have shown that both originate from a common precursor (24). Therefore, it is conceivable that the 88,000-dalton species in tunicamycin-treated controls is the same glycopeptide (gp88) found in untreated infected controls. This conclusion suggests that during posttranslational addition of oligosaccharides, some nascent polypeptides evade the addition of *N*-linked oligosaccharide but subsequently acquire *O*-linked side chains.

The difference in molecular weight between the final product (gp98) and the high-mannose precursor (gp81) is 17,000. During processing of *N*-linked oligosaccharides, the three terminal glucose residues and six of the nine mannose residues are removed. Glucosamine (three molecules per chain), sialic acid (0 to 3 molecules per chain), galactose (3 molecules per chain), and fucose (1 molecule per chain) are subsequently added to $GlcNAc_2Man_3$ by glycosyl transferases in the Golgi apparatus (31). Therefore, the processing of gp81 cannot account for the observed shift in molecular weight of approximately 17,000 daltons. We conclude, therefore, that gp98 contains both *N*-linked and *O*-linked oligosaccharides. Similar observations have been reported in herpes simplex virus type 1-infected cells treated with tunicamycin (41).

In the presence of the ionophore monensin, mature gp98 was not synthesized. Likewise, it has been shown that the transport of other enveloped virus membrane proteins from the Golgi apparatus to the plasma membrane can be blocked by monensin (2, 17, 18, 30). Concomitant with the block in transport is a failure of infected cells to complete processing of the glycoprotein oligosaccharide side chains. In the presence of monensin Friend murine leukemia virus envelope glycoproteins do not contain complex oligosaccharides (30). In contrast, the block in glycoprotein processing by vesicular stomatitis virus, Sindbis virus (2, 17), and herpes simplex virus type 1 (18) occurs after acquisition of Endo H resistance. Although we found that gp98 was not synthesized, all other glycoprotein intermediates found in untreated VZV-infected cells were observed in the treated cultures. Interestingly, treatment of the mature gp98 with neuraminidase reduced the M_r to 90,000, a value identical to that observed in monensin-treated cultures. Therefore, one of the effects of monensin was inhibition of sialation of gp98. Although similar observations have not been reported in other virus systems, Tartakoff et al. found that monensin prevented sialation of the major mouse histocompatibility antigen, H2 (35). Consistent with the observations of these authors, we conclude that the addition of sialic acid residues to gp98 occurs distal to the Golgi subsite in HMC defined by monensin. This interruption in glycoprotein processing also blocks the insertion of altered gp98 into the plasma membrane, inhibits the envelopment of the nucleocapsid within the cytoplasm, and virtually eliminates the production of infectious VZV virions.

ACKNOWLEDGMENTS

This research was supported by Public Health Service grant AI14604 from the National Institutes of Health and by an award from the Morrison Trust, San Antonio, Tex. E.A.M. is a recipient of a predoctoral minority fellowship from the American Society for Microbiology.

LITERATURE CITED

1. **Achong, B. G., and E. V. Meurisse.** 1968. Observations on the fine structure and replication of varicella virus in cultivated human amnion cells. J. Gen. Virol. **3**:305–309.
2. **Alonso, F. V., and R. W. Compans.** 1981. Differential effect of monensin on enveloped viruses that form at distinct plasma membrane domains. J. Cell Biol. **89**:700–705.
3. **Bergh, M. L. E., J. M. Gerrit, J. M. Hooghwinkel, and D. K. van den Eijnden.** 1983. Biosynthesis of the *O*-glycosidically linked oligosaccharide chains of fetuin. J. Biol. Chem. **258**:7430–7436.
4. **Beyer, T. A., J. I. Rearick, J. C. Paulson, J. P. Prieels, J. E. Sadler, and R. L. Hill.** 1979. Biosynthesis of mammalian glycoproteins. J. Biol. Chem. **254**:12531–12541.
5. **Bonner, W. N., and R. A. Laskey.** 1974. A film detection method for tritium-labeled proteins and nucleic acid in polyacrylamide gels. Eur. J. Biochem. **43**:83–88.
6. **Burnette, W. N.** 1981. "Western blotting:" electrophoretic transfer of proteins from sodium dodecyl sulfate—polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. Anal. Biochem. **112**:195–203.
7. **Cohen, G. H., D. Long, J. T. Matthews, M. May, and R. Eisenberg.** 1983. Glycopeptides of the type-common glycoprotein gD of herpes simplex virus types 1 and 2. J. Virol. **46**:679–689.
8. **Fittschen, C., R. T. Parmley, R. L. Austin, and W. M. Crist.** 1983. Vicinal glycol staining identifies secondary granules in human normal and Chediak-Higashi neutrophils. Anat. Rec. **205**:301–311.
9. **Friedrichs, W. E., and C. Grose.** 1984. Glycoprotein gp118 of varicella-zoster virus: purification by serial affinity chromatography. J. Virol. **49**:992–996.
10. **Grose, C.** 1980. The synthesis of glycoproteins in human melanoma cells infected with varicella-zoster virus. Virology **101**:1–9.
11. **Grose, C., D. P. Edwards, W. E. Friedrichs, K. A. Weigle, and W. L. McGuire.** 1983. Monoclonal antibodies against three major glycoproteins of varicella-zoster virus. Infect. Immun. **40**:381–388.
12. **Grose, C., D. P. Edwards, K. A. Weigle, W. E. Friedrichs, and W. L. McGuire.** 1984. Varicella-zoster virus-specific gp140: a highly immunogenic and disulfide-linked structural glycoprotein. Virology **132**:138–146.
13. **Grose, C., W. E. Friedrichs, and G. C. Smith.** 1983. Purification and molecular anatomy of the varicella-zoster virion. Biken J. **26**:1–15.
14. **Grose, C., D. M. Perrotta, P. A. Brunell, and G. C. Smith.** 1979. Cell-free varicella-zoster virus in cultured human melanoma cells. J. Gen. Virol. **43**:15–23.
15. **Hope, R. G., and H. S. Marsden.** 1983. Processing of glycoproteins induced by herpes simplex virus type 1: sulphation and nature of the oligosaccharide linkages. J. Gen. Virol. **64**:1943–1953.
16. **Hubbard, S. C., and R. J. Ivatt.** 1981. Synthesis and processing of asparagine-linked oligosaccharides. Annu. Rev. Biochem. **50**:555–583.
17. **Johnson, D. C., and M. J. Schlesinger.** 1980. Vesicular stomatitis virus and Sindbis virus glycoprotein transport to the cell surface is inhibited by ionophores. Virology **103**:407–424.
18. **Johnson, D. C., and P. G. Spear.** 1982. Monensin inhibits the processing of herpes simplex virus glycoproteins, their transport to the cell surface, and the egress of virions from infected cells. J. Virol. **43**:1102–1112.
19. **Johnson, D. C., and P. G. Spear.** 1983. *O*-linked oligosaccharides are acquired by herpes simplex virus glycoproteins in the golgi apparatus. Cell **32**:987–997.
20. **Leavitt, R., S. Schlesinger, and S. Kornfeld.** 1977. Tunicamycin inhibits glycosylation and multiplication of Sindbis and vesicular stomatitis viruses. J. Virol. **21**:375–385.
21. **McEver, R. P., J. U. Baenziger, and P. W. Majerus.** 1982. Isolation and structural characterization of the polypeptide subunits of membrane glycoprotein IIb-IIIa from human platelets. Blood **59**:80–85.
22. **Nakamura, K., A. S. Bhown, and R. W. Compans.** 1980. Glycosylation sites of influenza viral glycoproteins. Tryptic glycopeptides from the A/WSN (H_0N_1) hemagglutinin glycoprotein. Virology **107**:208–221.
23. **Nathenson, S. G., H. Uehara, and B. M. Ewenstein.** 1981. Primary structural analysis of the transplantation antigens of the murine H-2 major histocompatibility complex. Annu. Rev. Biochem. **50**:1025–1052.
24. **Okuno, T., K. Yaminishi, K. Shiraki, and M. Takahashi.** 1983. Synthesis and processing of glycoproteins of varicella-zoster virus as studied with monoclonal antibodies to VZV antigens. Virology **129**:357–368.
25. **Olden, K., R. M. Pratt, and K. M. Yamada.** 1978. Role of carbohydrates in protein secretion and turnover: effects of tunicamycin in the major cell surface glycoprotein of chick embryo fibroblasts. Cell **13**:461–473.
26. **Pizer, L. I., G. H. Cohen, and R. J. Eisenberg.** 1980. Effect of tunicamycin on herpes simplex virus glycoproteins and infectious virus production. J. Virol. **34**:142–153.
27. **Robbins, P. W., S. C. Hubbard, S. J. Turco, and D. F. Wirth.** 1977. Proposal for a common oligosaccharide intermediate in the synthesis of membrane glycoproteins. Cell **12**:893–900.
28. **Schauer, R.** 1978. Characterization of sialic acids. Methods Enzymol. **50C**:64–89.
29. **Schwarz, R. T., J. M. Rohrschneider, and M. F. G. Schmidt.** 1976. Suppression of glycoprotein formation of Semliki Forest, influenza, and avian sarcoma viruses by tunicamycin. J. Virol. **19**:782–791.
30. **Srinivas, R. V., L. R. Melsen, and R. W. Compans.** 1982. Effects of monensin on morphogenesis and infectivity of Friend murine leukemia virus. J. Virol. **42**:1067–1075.
31. **Strous, G. J. A. M., and H. F. Lodish.** 1980. Intracellular

transport of secretory and membrane proteins in hepatoma cells infected by vesicular stomatitis virus. Cell **22**:709–717.
32. **Tabas, I., S. Schlesinger, and S. Kornfeld.** 1978. Processing of high mannose oligosaccharides to form complex type oligosaccharides on the newly synthesized polypeptides of the vesicular stomatitis virus G protein and the IgG heavy chain. J. Biol. Chem. **253**:716–722.
33. **Tai, T., K. Yamashita, and A. Kobata.** 1977. The substrate specificities of endo-β-*N*-acetylglucosaminidases C_{II} and H. Biochem. Biophys. Res. Commun. **78**:434–441.
34. **Tarentino, A. L., T. H. Plummer, Jr., and F. Maley.** 1974. The release of intact oligosaccharides from specific glycoproteins by endo-β-*N*-acetylglucosaminidase H. J. Biol. Chem. **249**:818–824.
35. **Tartakoff, A., D. Hoessli, and P. Vassalli.** 1981. Intracellular transport of lymphoid surface glycoproteins. Role of the golgi complex. J. Mol. Biol. **150**:525–535.
36. **Tkacz, J. S., and J. O. Lampen.** 1975. Tunicamycin inhibition of polyisoprenyl n-acetylglucosaminyl pyrophosphate formation in calf-liver microsomes. Biochem. Biophys. Res. Commun. **65**:248–257.
37. **Towbin, H., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. U.S.A. **76**:4350–4354.
38. **Weigle, K. A., and C. Grose.** 1983. Common expression of varicella-zoster viral glycoproteins antigens in vitro and in chickenpox and zoster vesicles. J. Infect. Dis. **148**:630–638.
39. **Weigle, K. A., and C. Grose.** 1984. Molecular dissection of the humoral immune response to individual varicella-zoster viral proteins during chickenpox, quiescence, reinfection, and reactivation. J. Infect. Dis. **149**:741–749.
40. **Wenske, E. A., M. W. Bratton, and R. J. Courtney.** 1982. Endo-β-*N*-acetylglucosaminidase H sensitivity of precursors of herpes simplex virus type 1 glycoproteins gB and gC. J. Virol. **44**:241–248.
41. **Wenske, E. A., and R. J. Courtney.** 1983. Glycosylation of herpes simplex virus type 1 gC in the presence of tunicamycin. J. Virol. **46**:297–301.