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Varicella-Zoster Virus Glycoprotein Oligosaccharides Are Phosphorylated during Posttranslational Maturation

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Varicella-zoster virus (VZV)-infected human embryonic lung fibroblasts (HELF) do not release infectious virions into their growth medium. Extracellular virions are pleomorphic, suggesting that they are partially degraded before their release from cells. To examine the intracellular pathway of viral maturation, [2-^{3}H]mannose-labeled virus-encoded glycoproteins were isolated from VZV-infected HELF. Oligosaccharides attached to the glycoproteins were processed to complex-type units, some of which were phosphorylated. The major intracellular site of accumulation of VZV gpI was found to be perinuclear and to correspond to that of the cation-independent mannose 6-phosphate (Man 6-P) receptor. Subsets of VZV-containing cytoplasmic vacuoles were coated, Golgi-associated, or accessible to endocytic tracers. Phosphorylated monosaccharides protected HELF from the cytopathic effect of VZV in proportion to their ability to block Man 6-P receptor-mediated endocytosis. These data suggest that the unusual phosphorylated oligosaccharides mediate an interaction between VZV and Man 6-P receptors of the host cell; this interaction may be responsible for withdrawal of newly synthesized virions from the secretory pathway and for their diversion to prelysosomal structures.

Varicella-zoster virus (VZV) is the causative agent of both varicella (chickenpox) and zoster (shingles) infections (47). Fluid from the vesicular lesions of children with chickenpox contains cell-free virus that is capable of infecting cells in culture (43); however, the cultured cells do not in turn release infectious virus into the medium (47). Spread of the virus in culture, therefore, must rely upon the interaction of an infected cell with adjacent uninfected cells. Cell-free virus can be obtained from infected tissue culture cells only by mechanical disruption; the yield of infectious particles, however, is extremely low (39). In contrast to the strict cell-associated nature of VZV, cultured cells infected with other herpesviruses, such as herpes simplex virus (HSV), release infectious virions into the culture supernatant.

VZV particles accumulate intracellularly within large cytoplasmic vacuoles and at the surfaces of infected cells grown in tissue culture (5, 12, 20). The majority of both intracellular and extracellular virions appear pleomorphic when examined by electron microscopy; in most cases, the viral coat is disrupted and the central dense core is lost or is in disarray (5, 12). In contrast to VZV particles, HSV type 1 (HSV-1) virions generally appear intact and uniform within infected cells, and the extracellular particles retain their integrity (5). This differential behavior suggests that cells infected with VZV retain and degrade the viral particles intracellularly and release partially digested virions, whereas cells infected with HSV release intact infectious particles into the supernatant medium. The pleomorphic structure of cell-associated VZV suggests that the cytoplasmic vacuoles within which the virions accumulate contain hydrolytic enzymes that degrade the viral coat. Since the vacuoles that harbor VZV in human embryonic lung fibroblasts (HELFs) also contain acid phosphatase (12), it is likely that these cytoplasmic vacuoles contain components common to the lysosomal compartment.

Lysosomal acid hydrolases are synthesized in the rough endoplasmic reticulum (ER) and are delivered to the lysosomal compartment by the attachment of a specific recognition marker, mannose 6-phosphate (Man 6-P), to the asparagine-linked oligosaccharides of the enzymes (24, 27, 35, 36, 40, 46). This recognition marker facilitates binding of the acid hydrolases to a Man 6-P receptor and the specific diversion of the hydrolases from the secretory to the lysosomal pathway. In view of the previous suggestion that VZV accumulates within organelles that possess lysosomal enzymes (12), we examined the intracellular maturation process and characterized the cytoplasmic sites of virion accumulation. The results indicate that the viral glycoproteins contain phosphorylated complex-type oligosaccharides and that the intracellular vacuoles that contain virus particles also contain endosomal markers and the cation-independent (CI) Man 6-P receptor. VZV may remain cell associated, therefore, by a mechanism similar to that involved in the targeting of acid hydrolases to lysosomes.

MATERIALS AND METHODS

Cells and virus. HELFs (Whittaker M. A. Bioproducts, Walkersville, Md.) were grown as monolayers in 32-oz prescription bottles inoculated with a wild-type strain of VZV and incubated in Earle minimum essential medium (MEM) enriched with 2% fetal calf serum (Flow Laboratories, Alexandria, Va.) (maintenance medium). The fetal calf serum was inactivated for 30 min at 56°C. Infected monolayers were incubated at 35 to 37°C until 90% cytopathic effect (CPE) occurred, usually 48 h. Monolayers were then rinsed with Hanks balanced salt solution, scraped with a rubber policeman into 1.5 ml of Hanks balanced salt solution per prescription bottle, transferred to a 15-ml centrifuge tube, and sonicated (Branson sonicator) for 30 s at 2.2 to 2.6

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A in an ice bath. The solution was centrifuged at low speed to remove intact cells and debris; supernatants were used as cell-free virus; samples were stored at −70°C until ready to use. Phosphorylated hexoses as specified were added to some samples of cell-free virus at a final concentration of 20 mM before being frozen.

Inhibition of viral infection with monosaccharides. HELF monolayers were propagated in tubes (16 by 125 mm) and when confluent were treated for 30 min with Man 6-P, glucose 6-phosphate (Glu 6-P), or glucose 1-phosphate (Glu 1-P) at concentrations ranging from 1 to 20 mM; dilutions were made in maintenance medium. For a control, cell-free virus in maintenance medium alone was used. When a culture contained a phosphorylated hexose, the monosaccharide was present at the indicated concentration during all phases of adsorption and growth. An inoculum of 0.2 ml of cell-free virus (about 50 to 100 PFU/ml) (in quadruplicate) was added, and the tubes were incubated at 35 to 37°C. Two days later, the cultures were trypsinized and passed to four fresh HELF monolayers to increase the multiplicity of infection. Two days later, the number of infectious centers in the four cultures was counted.

Electron microscopy. Confluent monolayers of HELFs on ACLAR disks (Allied Fibers and Plastics, Pottsville, Pa.) were seeded with VZV-infected HELFs and incubated at 37°C for 24 h. To mark the lysosomal compartment, the cells were preincubated with horseradish peroxidase (HRP; 1 mg/ml in MEM–10% fetal calf serum) for 24 h before addition of the infected cells; HRP was removed from the medium during the infection. HRP enters cells via pinocytosis and accumulates within lysosomes in which it is slowly degraded (41). Of the HRP activity associated with the HELF monolayer after the initial loading, 39% persisted after a subsequent 24-h chase, and the enzyme activity cofractionated with lysosomes when an extract of the HRP-containing cells was separated by Percoll density gradient centrifugation (data not shown). Alternatively, to mark the endosomal compartment, the VZV-infected monolayer (24 h after addition of the infected cells) was incubated in medium containing 1 mg of HRP per ml for 60 min. In both protocols, the VZV-infected HRP-containing monolayer was fixed for 30 min at room temperature in 0.1 M phosphate (pH 7.3) containing 2% glutaraldehyde, 120 mM glucose, and 0.4 mM $CaCl_2$. The fixed monolayers were rinsed with 0.1 M phosphate (pH 7.3) and then incubated in 3,3′-diaminobenzidine (DAB) substrate mixture (0.1 M phosphate [pH 7.3] containing 0.6 mg of DAB per ml, 2 mg of glucose per ml, 0.4 mg of NH_4Cl per ml, and 3 μg of glucose oxidase per ml) for 15 to 30 min at room temperature. The monolayers were rinsed with 0.1 M phosphate (pH 7.3), postfixed with 1% OsO_4 in 0.1 M phosphate, (pH 7.3), and then dehydrated in a graded series of ethanols, cleared in propylene oxide, and embedded in Epon 812. Thin sections (silver grey) were cut, stained with uranyl acetate and lead citrate, and examined with a JOEL EM1200 electron microscope.

Immunofluorescence microscopy. Confluent monolayers of HELFs on ACLAR disks were seeded with VZV-infected HELFs. After 24 h at 37°C, the monolayers were fixed in either 4% paraformaldehyde or in lysine-periodate-formadehyde as previously detailed (28). Results were comparable with either fixative, but the fluorescence was brighter using the latter fixative. The fixed cells were extracted at room temperature for 30 min in 0.1 M phosphate (pH 7.3) containing 0.2% Triton X-100–4% horse serum. The cells subsequently were incubated with a rabbit polyclonal antiserum prepared against bovine liver CI Man 6-P receptor and a mouse monoclonal antibody against VZV gpI (a gift of Ronald Ellis, Merck Research Laboratories, West Point, Pa.). The anti-receptor antibody was detected by using biotinylated goat anti-rabbit immunoglobulin G (Kirkegard and Perry, Gaithersburg, Md.) and avidin-Texas red (Bethesda Research Laboratories, Inc., Bethesda, Md.), and the mouse monoclonal antibody was visualized by using fluorescein isothiocyanate-labeled goat anti-mouse immunoglobulin G (Boehringer Mannheim Biochemicals, Indianapolis, Ind.).

Metabolic labeling. VZV-infected HELF monolayers (18 h postinfection) were rinsed with glucose-free MEM containing 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) (pH 7) and 10% dialyzed fetal bovine serum (glucose-free medium). Each 150-cm^2 flask received 12 ml of glucose-free medium containing 80 μCi/ml of [2-^{3}H]mannose (15 Ci/mmol; American Radiolabel Company, St. Louis, Mo.), and the cells were incubated for 30 min at 37°C. After the labeling, the pulse medium was removed and 30 ml of complete α-MEM (containing 20 mM HEPES [pH 7], 10% fetal bovine serum) was added to initiate the chase; the chase medium was preequilibrated to 20 or 37°C, depending on the experiment. After the chase, the monolayer was rinsed once with cold phosphate-buffered saline containing 2 mg of bovine serum albumin per ml (PBS/BSA), and the cells were scraped into PBS/BSA with a rubber policeman and collected by centrifugation. The cell pellets were frozen immediately in a dry ice-ethanol bath and stored at −70°C.

Immunoprecipitation. Cell pellets were suspended in 1 ml of 25 mM HEPES (pH 7)–0.1 M NaCl–5 mM β-glycerophosphate–1% Triton X-100–0.2% deoxycholate–5 mM Man 6-P (extraction buffer). The extract was incubated for 30 min on ice and was then clarified at 10,000 × *g* for 15 min. The supernatant was set aside, and the pellet was suspended in 1 ml of extraction buffer by sonication. The resuspended pellet and the original supernatant were combined and centrifuged for 30 min at 12,000 × *g*; the resulting pellet was discarded. The extract was precleared by the addition of 0.15 ml of 10% Pansorbin (Calbiochem-Behring, San Diego, Calif.); after 30 min on ice the bacteria were removed by centrifugation, and 0.04 ml of human convalescent zoster serum was added to the supernatant. Immune complexes were allowed to form for 2 h at 4°C, after which they were recovered by the addition of 0.4 ml of 10% Pansorbin. After 30 min on ice, the bacteria were collected by centrifugation and washed five times with 25 mM HEPES (pH 7)–0.1 M NaCl–10 mM β-glycerophosphate–1% Triton X-100–0.1% deoxycholate–0.1 mM phenylmethylsulfonyl fluoride and once with 50 mM Tris (pH 6.8) (2 ml per wash). The final pellet was suspended in 0.4 ml of sodium dodecyl sulfate (SDS) sample buffer (26) by sonication, the mixture was boiled for 3 min, and the bacteria were removed by centrifugation. The disaggregated immunoprecipitates were then fractionated by SDS gel electrophoresis (using a 7.5 or 10% polyacrylamide gel). The gels were soaked in Amplify (Amersham Corp., Arlington Heights, Ill.) before being dried; fluorography was performed at −70°C.

Fractionation of ^{3}H-labeled glycopeptides. The regions of the dried gel containing the ^{3}H-labeled VZV glycoproteins were excised, and the radioactivity was solubilized by digesting the gel slices with 5 mg of pronase in 1 ml of 0.1 M Tris (pH 8)–20 mM $CaCl_2$–0.2 M β-glycerophosphate. The digests were incubated overnight at 56°C, boiled for 3 min, diluted with 4 ml of H_2O, and clarified by centrifugation. The resulting supernatants were dried by rotary evaporation under reduced pressure. The glycopeptides were desalted by Sephadex G-25 chromatography and fractionated on conca-

navalin A (ConA)-Sepharose; bound glycopeptides were recovered by sequential elution with 10 mM α-methylglucoside (peak II) and 100 mM α-methylmannoside (peak III) as previously described (7). Elution of the glycopeptides were monitored by liquid scintillation counting, and the appropriate fractions were pooled, dried, and desalted.

For glycopeptidase F digestion, the VZV glycoproteins were immunoprecipitated from VZV-infected HELFs after a 30-min pulse at 37°C and a 3-h chase at 20°C. The disaggregated immunoprecipitate was chromatographed on a Sephadex G-50 column (1.5 by 50 cm), equilbrated in 20 mM Tris (pH 8)–10 mM β-glycerophosphate–0.2% SDS (column buffer), and the excluded glycoproteins (fractions 18 to 22) were lyophilized. The dried residue was dissolved in 0.8 ml of H_2O, 200 μg of hemoglobin was added as carrier, and the proteins were precipitated by the addition of 10 volumes of cold acetone. The resulting precipitate was collected by centrifugation and washed sequentially with 10% trichloroacetic acid and acetone; the washed pellet was suspended in 0.3 ml of 0.1 M phosphate (pH 7.5)–10 mM EDTA–0.2% SDS–1% β-mercaptoethanol and boiled for 3 min. After the addition of 0.016 ml of 20% Triton X-100, 0.01 ml of glycopeptidase F (1 U; Boehringer Mannheim) was added and the digest was incubated overnight at 37°C. The reaction mixture was diluted with 0.7 ml of column buffer and reapplied to the Sephadex G-50 column. Released oligosaccharides were pooled (22 ml), and 0.3 ml of 2 M KCl was added to precipitate the SDS. After centrifugation, the supernatant was dried by rotary evaporation, and the oligosaccharides were desalted by Sephadex G-25 chromatography and fractionated on ConA-Sepharose.

Structural analysis. Strong acid hydrolysis was accomplished by dissolving the glycopeptides (or oligosaccharides) in 0.3 ml of 2 N HCl and heating the mixture at 100°C for 4 h. The hydrolysates were dried by rotary evaporation, dissolved in H_2O, and spotted onto Whatman no. 1 paper. The chromatograms were developed by descending chromatography overnight, using ethyl acetate-pyridine-acetic acid-H_2O (5:5:1:3) as solvent. The dried chromatograms were cut into 1-cm strips, and the associated radioactivity was determined by liquid scintillation counting. Samples treated with alkaline phosphatase were dissolved in 0.03 ml of 50 mM Tris (pH 8) containing 0.2 U of *Escherichia coli* alkaline phosphatase (Calbiochem-Behring). The digests were incubated overnight at 37°C and then diluted with 1 ml of H_2O and applied to 1-ml columns of Dowex 50 (H^+); the columns were eluted with 5 ml of H_2O. The eluates were dried, and the samples were analyzed by descending paper chromatography.

RESULTS

Analysis of the posttranslational maturation of VZV glycoproteins within HELFs. To characterize the maturation of newly synthesized viral glycoprotein-associated oligosaccharides, VZV-infected HELF monolayers were labeled with [2-^{3}H]mannose and virion-specific glycoproteins were immunoprecipitated from cell extracts, using a human convalescent zoster serum. Four major labeled proteins were recovered in the immunoprecipitate after a 30-min pulse (Fig. 1, lanes 1 and 2). The apparent molecular sizes of these proteins corresponded to those expected (18, 29, 31, 33) for the precursors of gpII (pgpII, 120 kilodaltons [kDa]), gpIII (pgpIII, 102 kDa), gpI (pgpI, 77 kDa), and gpIV (pgp IV, 52 kDa). After a 6-h chase, the total amount of radioactivity recovered in the immunoprecipitate declined, and the major species corresponded to the mature forms of gpIII (118 kDa), gpI (98 kDa), gpII (66 kDa), and gpIV (60 kDa) (Fig. 1, lanes 3 and 4).

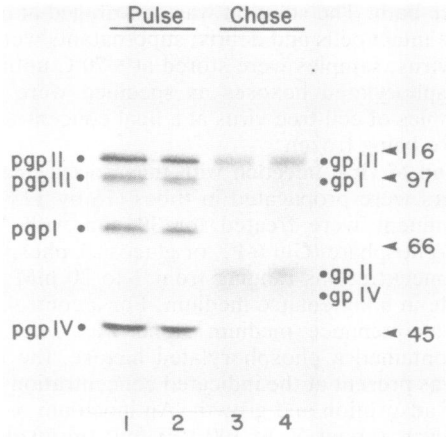


FIG. 1. Pulse-chase analysis of VZV-encoded glycoproteins. VZV-infected HELFs were pulse-labeled for 30 min with [2-^{3}H]mannose and harvested immediately (lanes 1 and 2) or after 3 h of chase at 37°C (lanes 3 and 4). The viral glycoproteins were immunoprecipitated from detergent extracts of the cells and analyzed by SDS gel electrophoresis and fluorography. The migration positions of molecular weight standards are indicated by the arrowheads on the right, and the identities of the ^{3}H-labeled VZV glycoproteins are denoted next to lanes 1 and 4 of the fluorogram. The identity of the radiolabeled species in lanes 3 and 4 between gpI and gpII is unknown.

Glycopeptides generated by pronase digestion of the radioactive regions of the dried gel shown in Fig. 1 were separated on ConA-Sepharose into three fractions corresponding to tri- and/or tetraantennary complex-type units (peak I), biantennary complex-type units (peak II), and high-mannose-type structures (peak III). Radiolabeled glycopeptides recovered from the precursor molecules (Fig. 2, lanes A through D) eluted predominantly as high-mannose-type structures; this is to be expected, since glycoproteins are cotranslationally glycosylated by the addition of a preformed high-mannose-type unit (25). In contrast, the majority of the ^{3}H-labeled glycopeptides recovered from the mature forms of the glycoproteins eluted as complex-type structures (Fig. 2, panels E through H). The apparent loss of radioactivity between the pulse-labeled and mature species detected in Fig. 1, therefore, is due, in part, to the loss of [^{3}H]mannose residues during the conversion of high-mannose-type units to complex-type species. Since the posttranslational conversion of a high-mannose oligosaccharide to a complex-type unit occurs in the Golgi apparatus (25), the majority of the viral glycoproteins passed through this organelle during their maturation.

The lysosomal acid hydrolase Man 6-P recognition marker is specifically attached to high-mannose-type asparagine-linked oligosaccharides (24, 32, 45). To determine whether the viral glycoproteins contained similar Man 6-P-containing structures, the high-mannose-type oligosaccharides were degraded by acid hydrolysis and the resulting monosaccharides were separated by descending paper chomatography. Man 6-P was not detected as a constituent of the high-mannose oligosaccharides recovered from the precursor or mature forms of the viral glycoproteins. Since acid hydrolases are maximally phosphorylated within 60 min of their synthesis and are subsequently dephosphorylated after

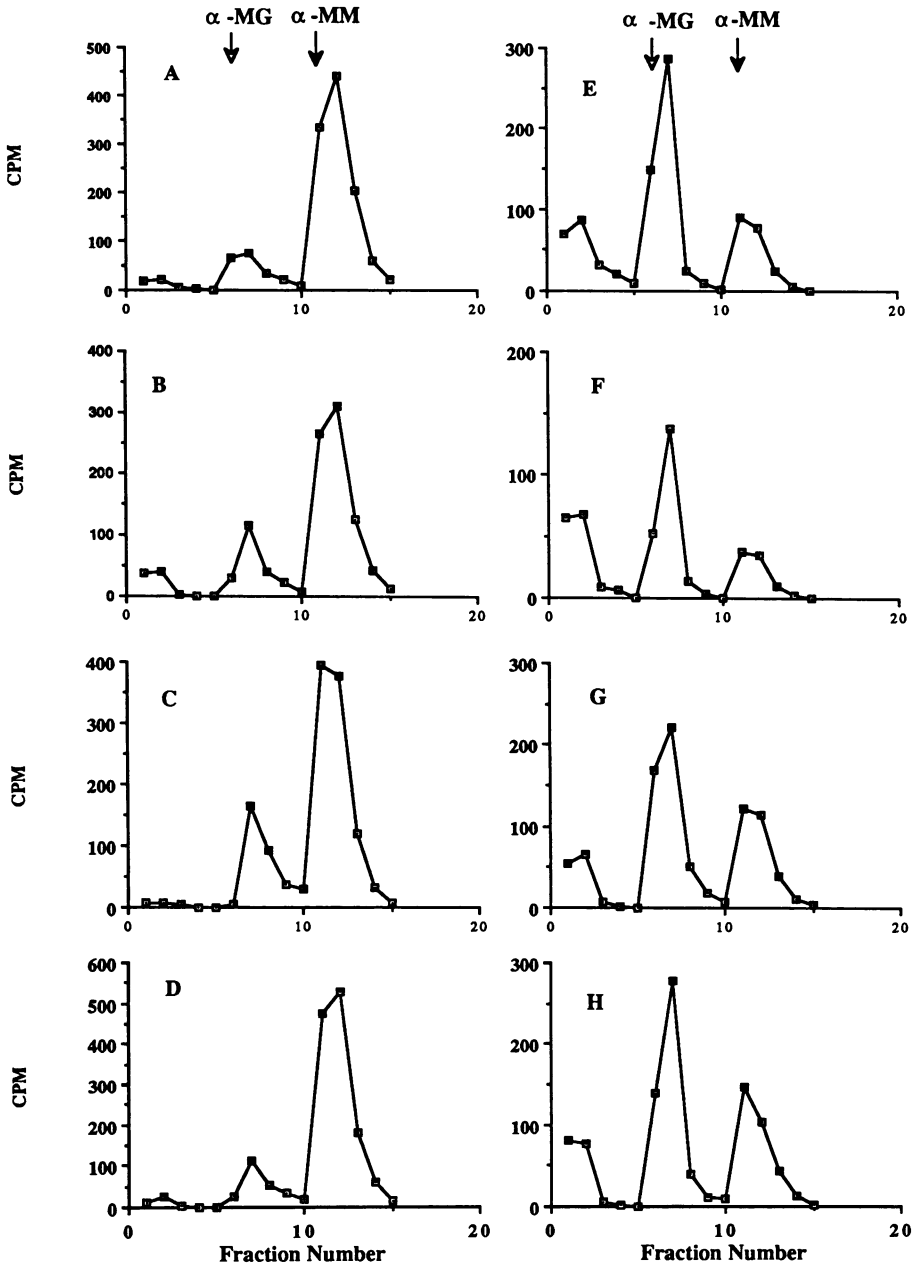


FIG. 2. ConA-Sepharose chromatography of [^{3}H]mannose-labeled VZV glycopeptides. The radioactive regions of the dried gel shown in Fig. 1 were excised, and the radioactivity was solubilized by pronase digestion. The resulting glycopeptides were applied to ConA-Sepharose columns, and bound glycopeptides were eluted by the sequential addition of 10 mM α-methylglucoside (α-MG) and 100 mM α-methylmannoside (α-MM). The profiles show the distribution of radioactivity recovered from pgpII (A), pgpIII (B), pgpI (C), and pgpIV (D) after the 30-min pulse and from gpIII (E), gpI (F), gpII (G), and gpIV (H) after the 3-h chase.

reaching lysosomes (11), the viral glycoproteins were isolated from VZV-infected HELFs after a 30-min pulse and 60-min chase to ensure that a transient phosphorylation did not occur. Again, Man 6-P was not detected in the hydrolysates of the high-mannose-type glycopeptides. The hydrolysate of the gpI oligosaccharides, for example, contained only radiolabeled mannose residues (Fig. 3B). For comparison, the acid hydrolysate of a purified one phosphomonoester-containing high-mannose oligosaccharide isolated from a mixture of acid hydrolases is shown in Fig. 3A. The phosphorylated oligosaccharides yielded two peaks of radioactivity that comigrated with Man 6-P and mannose standards; the ratio of mannose to Man 6-P was 7.1 to 1, indicating that one of eight mannose residues was phosphorylated.

Although complex-type asparagine-linked oligosaccharides attached to acid hydrolases are not phosphorylated, some forms of thyroglobulin contain Man 6-P in association with complex-type structures (19). The composition of the viral complex-type glycopeptides, therefore, was determined. After acid hydrolysis, peak II glycopeptides isolated from gpI yielded radiolabeled mannose and fucose residues but no Man 6-P (Fig. 3D); complex-type oligosaccharides often are fucosylated, and [2-^{3}H]mannose is metabolized to fucose (23). The peak I glycopeptides, on the other hand,

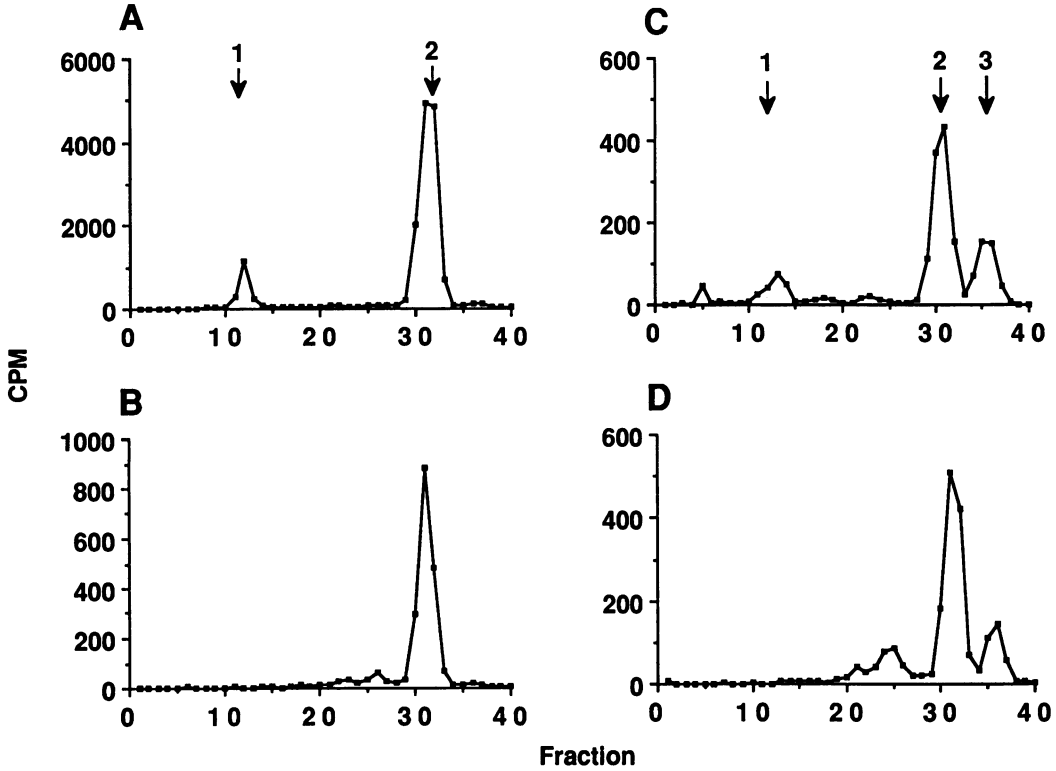


FIG. 3. gpI glycopeptides contain mannose phosphate residues. Glycopeptides generated from gpI after a 30-min pulse and 1-h chase of VZV-infected HELFs were fractionated on ConA-Sepharose. Units recovered in peaks I (C), II (D), and III (B) were degraded by acid hydrolysis, and the constituent monosaccharides were separated by descending paper chromatography. The hydrolysate of a one phosphomonoester-containing high-mannose oligosaccharide isolated from [^{3}H]mannose-labeled acid hydrolases is shown in panel A. The arrows in panels A and C show the migration of Man 6-P (peak 1), mannose (peak 2), and fucose (peak 3) standards.

yielded three peaks of radioactivity that corresponded to Man 6-P, mannose, and fucose (Fig. 3C); the mannose to Man 6-P ratio was 5.4 to 1. Thus, gpI contained mannose phosphate residues, but the type of oligosaccharide that was phosphorylated on the viral glycoprotein was different from the high-mannose-type units found on acid hydrolases.

To verify that the radioactive component migrating as Man 6-P contained a phosphomonoester, the peak I glycopeptide fraction was treated with alkaline phosphatase before and after acid hydrolysis. The posthydrolysis treatment led to a complete loss of the radioactivity that migrated as Man 6-P (Table 1). The [^{3}H]mannose-labeled component, therefore, contained a phosphomonoester group, and the resistance of the monoester to acid hydrolysis suggested that the phosphate was attached to the C-6 position of the mannose residue (3). The prehydrolysis treatment of the glycopeptides with alkaline phosphatase also reduced the amount of phosphorylated monosaccharide (Table 1). The phosphate, therefore, must exist as a monoester within the intact oligosaccharide and not as a diester which would be resistant to alkaline phosphatase.

TABLE 1. Sensitivity to alkaline phosphatase[a]

Condition	Mannose phosphate recovered (cpm)	% Control
No treatment	186	100
Posthydrolysis	0	0
Prehydrolysis	19	10

[a] Glycopeptides recovered from gpIII after a 30-min pulse and 1-h chase at 37°C were treated with alkaline phosphatase either before (prehydrolysis) or after (posthydrolysis) acid hydrolysis, and the amount of radioactivity migrating as Man 6-P was determined by paper chromatography. The control (no treatment) was hydrolyzed and analyzed without alkaline phosphatase digestion.

Formation of phosphorylated oligosaccharides at 20°C. As noted earlier, VZV glycoproteins are synthesized as precursor molecules which are subsequently processed to mature forms within infected cells. [^{3}H]mannose-labeled VZV-infected HELFs were chased at 20°C to slow the maturation process. The same four glycoprotein species detected in Fig. 1 were immunoprecipitated from the cells after a 30-min pulse-labeling at 37°C (Fig. 4). The viral glycoproteins underwent their expected maturations when the cells were chased at 37°C (Fig. 4, lanes 4 and 5), but at 20°C the precursor forms persisted (Fig. 4, lanes 2 and 3). For example, pgpI and pgpIV were totally absent after 3 h of chase at 37°C (lane 5), but these were the major species recovered after the 20°C chase (lane 3). Conversely, the mature forms of gpII and gpIV were the major proteins recovered after 3 h of chase at 37°C, but few of these proteins were produced at 20°C (compare lanes 3 and 5). The lower chase temperature, therefore, inhibited the conversion of the precursor glycoproteins to their mature counterparts.

Glycopeptides prepared from proteins immunoprecipitated after the 20°C chase were fractionated by ConA-Sepharose chromatography. As expected, the precursor glycoproteins contained predominantly high-mannose-type oligosaccharides (Table 2). At the lower temperature, therefore, many of the proteins did not reach the Golgi apparatus

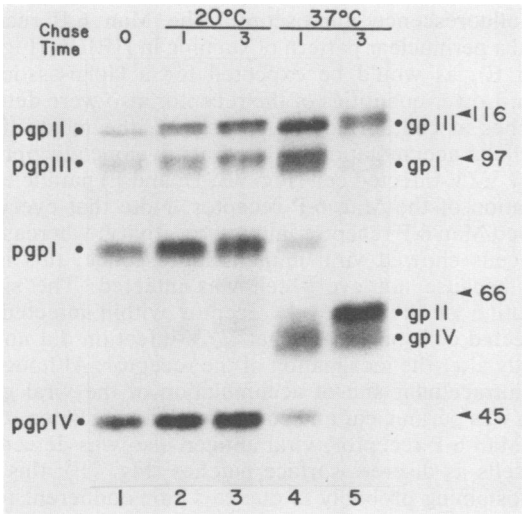


FIG. 4. Maturation of [^{3}H]mannose-labeled VZV glycoproteins is inhibited at 20°C. Monolayers of VZV-infected HELFs were pulse-labeled for 30 min at 37°C and chased at either 20 or 37°C. VZV-encoded glycoproteins were immunoprecipitated from detergent extracts of the cells after the pulse (lane 1) and after 1 (lane 2) and 3 (lane 3) h of chase at 20°C and after 1 (lane 4) and 3 (lane 5) h of chase at 37°C. The migration positions of molecular weight standards are indicated by the arrowheads at the right, and the identities of the ^{3}H-labeled VZV glycoproteins is denoted next to lanes 1 and 5 of the fluorogram.

and their oligosaccharides were not processed to complex-type units (25). Glycopeptides recovered from the region of the gel containing the mature forms of gpII and gpIV (it was impossible to distinguish the two proteins from one another on the gel after the 20°C chase) contained a larger percentage of complex-type units. Despite the impaired transport, each viral glycoprotein yielded glycopeptides that did not bind to ConA-Sepharose, and the mannose phosphate content of these structures was similar; the phosphorylated hexose accounted for 11 to 15% of the radioactivity (Table 2). Overall, the gpII-gpIV species contained the highest content of mannose phosphate (3.3%), whereas the precursor forms contained two to three times less of the phosphorylated hexose.

Nature of the phosphorylated oligosaccharides. The failure of the phosphorylated viral glycopeptides to bind to ConA-Sepharose suggested that the oligosaccharides were composed of tri- and/or tetraantennary asparagine-linked units (7). If O-linked glycopeptides were present, however, they also would not bind to ConA-Sepharose (8). To determine whether the mannose phosphate residue was associated with an N- or an O-linked oligosaccharide, the viral glycopeptides were analyzed as follows. First, the ConA-Sepharose peak I glycopeptides isolated from gpII were treated with mild base under conditions that release O-linked oligosaccharides from their peptide linkages (8). The alkaline hydrolysis did not alter the elution of the ^{3}H-labeled oligosaccharides from a Bio-Gel P6 column, suggesting that they were resistant to this treatment (data not shown). Second, the viral glycoproteins were digested with glycopeptidase F, an enzyme that removes most N-linked oligosaccharides from glycoproteins (42). Before digestion, the [^{3}H]mannose-labeled VZV glycoproteins were excluded on a Sephadex G-50 column (Fig.

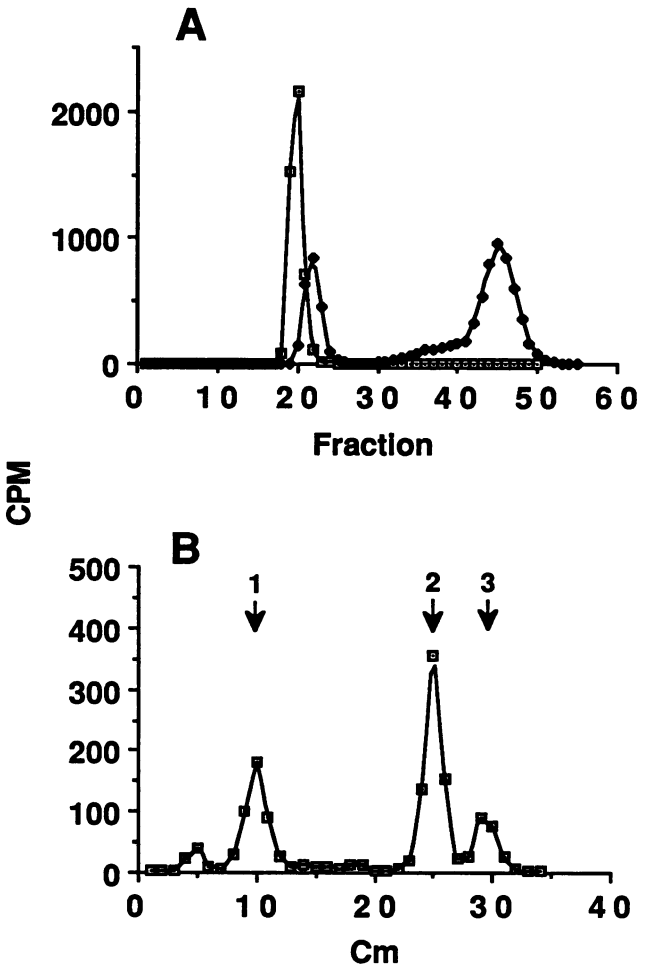


FIG. 5. VZV phosphorylated oligosaccharides are sensitive to glycopeptidase F. (A) VZV-infected HELFs were labeled with [^{3}H]mannose for 30 min at 37°C and then chased in isotope-free medium at 20°C for 3 h. [^{3}H]mannose-labeled VZV glycoproteins recovered by immunoprecipitation from the cell extract were chromatographed on Sephadex G-50 (⊡). The excluded glycoproteins were digested with glycopeptidase F and rechromatographed on the Sephadex G-50 column (◆). (B) The released oligosaccharides were fractionated on ConA-Sepharose, and those recovered in peak I were degraded by acid hydrolysis; the resulting monosaccharides were separated by descending paper chromatography. The arrows indicate the migration of Man 6-P (peak 1), mannose (peak 2), and fucose (peak 3) standards.

TABLE 2. Mannose phosphate content of VZV glycoproteins[a]

Glycoprotein species	Total (cpm, 10^3)	% in ConA-Sepharose peak			% of Peak I as Man 6-P	Overall % as Man 6-P
		I	II	III		
pgpII	42.2	7	6	87	12.8	0.9
pgpIII	36.6	15	6	79	11.4	1.7
pgpI	36	4	4	92	12	0.5
gpII-gpIV	29.4	24	13	63	13.7	3.3
pgpIV	51.8	4	2	94	14.8	0.6

[a] VZV-infected HELFs were labeled for 30 min at 37°C with [2-^{3}H]mannose and then chased for 3 h at 20°C. Glycopeptides were generated from the individual glycoproteins after immunoprecipitation and fractionated on ConA-Sepharose. The peak I glycopeptides were subsequently degraded by acid hydrolysis, and the quantity of radioactivity migrating as Man 6-P was determined by paper chromatography.

5A). In contrast, after treatment of the excluded glycoproteins with glycopeptidase F, 70% of the radioactivity eluted near the included volume of the column (Fig. 5A). The released oligosaccharides were fractionated on ConA-Sepharose, and those that eluted in peak I were degraded by acid hydrolysis. The glycopeptidase-released oligosaccharides yielded monosaccharides that comigrated with Man 6-P, mannose, and fucose (Fig. 5B); the ratio of mannose to Man 6-P was 1.9 to 1. Since the phosphorylated oligosaccharides were released by glycopeptidase F and were resistant to alkaline hydrolysis, the phosphorylated residues are assumed to be components of asparagine-linked oligosaccharides. At present, the precise structure of the phosphorylated oligosaccharides is unknown.

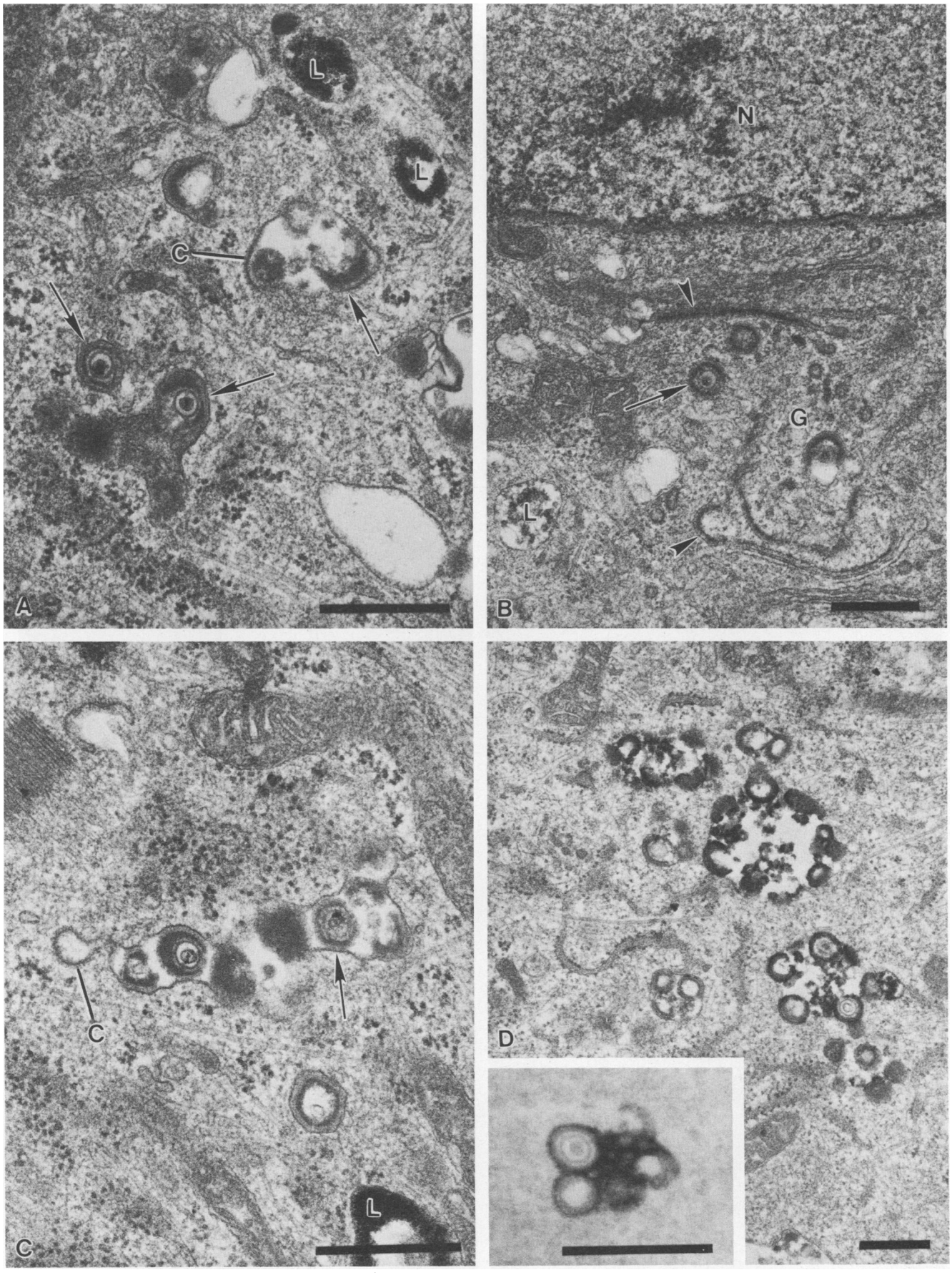
Characterization of the cytoplasmic site of VZV accumulation. Since the viral glycoproteins contained phosphorylated oligosaccharides, we compared the relationship of virion-containing vacuoles in VZV-infected HELFs with organelles known to be components of the lysosomal enzyme transport pathway. Noninfected HELFs were incubated with HRP for 24 h, after which fresh medium was added and the cells were infected with VZV. HRP enters cells via fluid-phase pinocytosis and accumulates within lysosomes (41). The infected monolayer was fixed, incubated with DAB and an H_2O_2-generating system, and processed for electron microscopy. The DAB reaction product that signified the location of HRP was evident within vacuoles located throughout the cytoplasm (Fig. 6A); these structures possessed the expected appearance of lysosomes. With few exceptions, however, the cytoplasmic vacuoles that contained the pleomorphic virions did not contain the DAB reaction product (Fig. 6A and 6C). Vesicles that contained recognizable virions or viral envelopes often were seen in close proximity to the Golgi apparatus (Fig. 6B), and many of these were coated (Fig. 6A C). To determine whether the virion-laden vesicles were common to the endocytic compartment, HELFs that had been infected with VZV 24 h previously were incubated for 60 min with HRP and immediately processed for electron microscopy. The DAB reaction product again was observed in cytoplasmic vacuoles; however, many of these DAB-labeled vesicles now contained virions (Fig. 6D). Other membrane-enclosed cytoplasmic structures contained virions or viral envelopes and remained devoid of the DAB reaction product (not shown).

The inability of HRP to accumulate within many of the virion-containing cytoplasmic vacuoles suggested that these structures were neither endosomes nor lysosomes; however, since the virion-containing structures displayed regions of coated membranes as has been observed for the Golgi apparatus (6, 17), it was possible that they were derived from this organelle. In many cells the CI Man 6-P receptor is located predominantly within the *trans*-Golgi reticulum and endosomal compartments in which it functions in the transport of newly synthesized acid hydrolases (13, 16). The distribution of a viral envelope glycoprotein (gpI), therefore, was compared with that of the CI Man 6-P receptor by immunofluorescence microscopy. The Man 6-P receptor showed a perinuclear pattern of staining in HELFs (Fig. 7A, C, and E), as would be expected for a Golgi-associated protein. Lower quantities of the receptor also were detected in patches at cell surfaces. Importantly, the major site of intracellular accumulation of the viral envelope glycoprotein in every VZV-infected cell (Fig. 7B, D, and F) paralleled the distribution of the Man 6-P receptor. Note that every cell displayed Man 6-P receptor immunoreactivity, whereas only a few cells showed viral immunofluorescence; this is expected because not every cell was infected. The similar distribution of the Man 6-P receptor within infected and noninfected cells suggested that VZV-infection did not significantly alter the localization of the receptor. Although the major intracellular site of accumulation of the viral glycoprotein was perinuclear and corresponded in location to that of the Man 6-P receptor, viral antigen also was detected in many cells as discrete surface patches (Fig. 7F); this viral immunostaining probably is due to virions adherent to the plasma membrane (5, 12). Note, however, that perinuclear viral immunostaining corresponds to that of the Man 6-P receptor even in cells that lack evidence of surface virions (Fig. 7A through D). The major intracellular site of viral antigen accumulation, therefore, coincides with the distribution of the Man 6-P receptor.

Inhibition of VZV-induced CPE. Monolayers of HELFs infected with VZV developed plaques as a result of localized areas of cell toxicity (Fig. 8A). When the monolayer was infected with cell-free virus in the presence of 20 mM Man 6-P, the CPE was reduced markedly (Fig. 8B). The protection afforded by Man 6-P was significant relative to control cultures (Fig. 9A; $P < 0.001$). At the same concentration, Glu 6-P was less effective than Man 6-P ($P < 0.001$), and Glu 1-P produced even less inhibition than did Glu 6-P (Fig. 9A; $P < 0.01$). The inhibition by Man 6-P was concentration dependent, and at 2.5 mM the phosphorylated hexose still provided significant protection against the virus-induced CPE (Fig. 9B; $P < 0.001$). In contrast to Man 6-P, Glu 6-P at 2.5 mM failed to inhibit VZV-induced CPE; significant inhibition was not encountered below a concentration of 10 mM ($P < 0.05$). At all concentrations tested (2.5 to 20 mM), Man 6-P produced a greater degree of inhibition than did Glu 6-P or Glu 1-P ($P < 0.001$). Thus, the phosphorylated monosaccharides protected HELF monolayers from the CPEs of VZV. Man 6-P was significantly more effective than Glu 6-P or Glu 1-P, and this selectivity was comparable to that observed with respect to the ability of phosphorylated monosaccharides to inhibit internalization of acid hydrolases via the Man 6-P receptor (21). HELF monolayers incubated with ^{125}I-labeled Man 6-P-bearing acid hydrolases internalized the radiolabeled ligands in a Man 6-P inhibitable process (not shown), as would be expected for cells that contain functional Man 6-P receptors at their surfaces (10).

Chloroquine was added to HELF cultures for 4 h, after which the cells were challenged with cell-free VZV. Both 25 and 50 μM concentrations of the weak base completely

FIG. 6. Localization of cytoplasmic VZV in relation to a lysosomal and an endosomal tracer. (A to C) In order to label lysosomes, monolayers of HELFs were incubated in medium containing HRP (1 mg/ml) for 24 h. The HRP was then removed, and the cells were infected with VZV. After 1 additional day of incubation, the monolayer was processed for electron microscopic visualization of peroxidase activity. Most HRP-containing cytoplasmic vacuoles (L) did not contain virions. Pleomorphic virions or viral envelope material was found in separate vesicles (arrows), some of which (panel B) appeared to be associated with the Golgi apparatus (G); some of these vesicles were coated (C; panels A and C). Apparent viral envelope material also appeared to accumulate in Golgi cisternae (arrowheads; panel B). (D) To label endosomes, VZV-infected HELFs were incubated for 1 h with HRP. Colocalization of virions and tracer in the same cytoplasmic compartment was found. (Inset) Unstained electron micrograph demonstrating HRP and VZV in the same cytoplasmic vesicle. N, Nucleus. Bars, 0.5 μm.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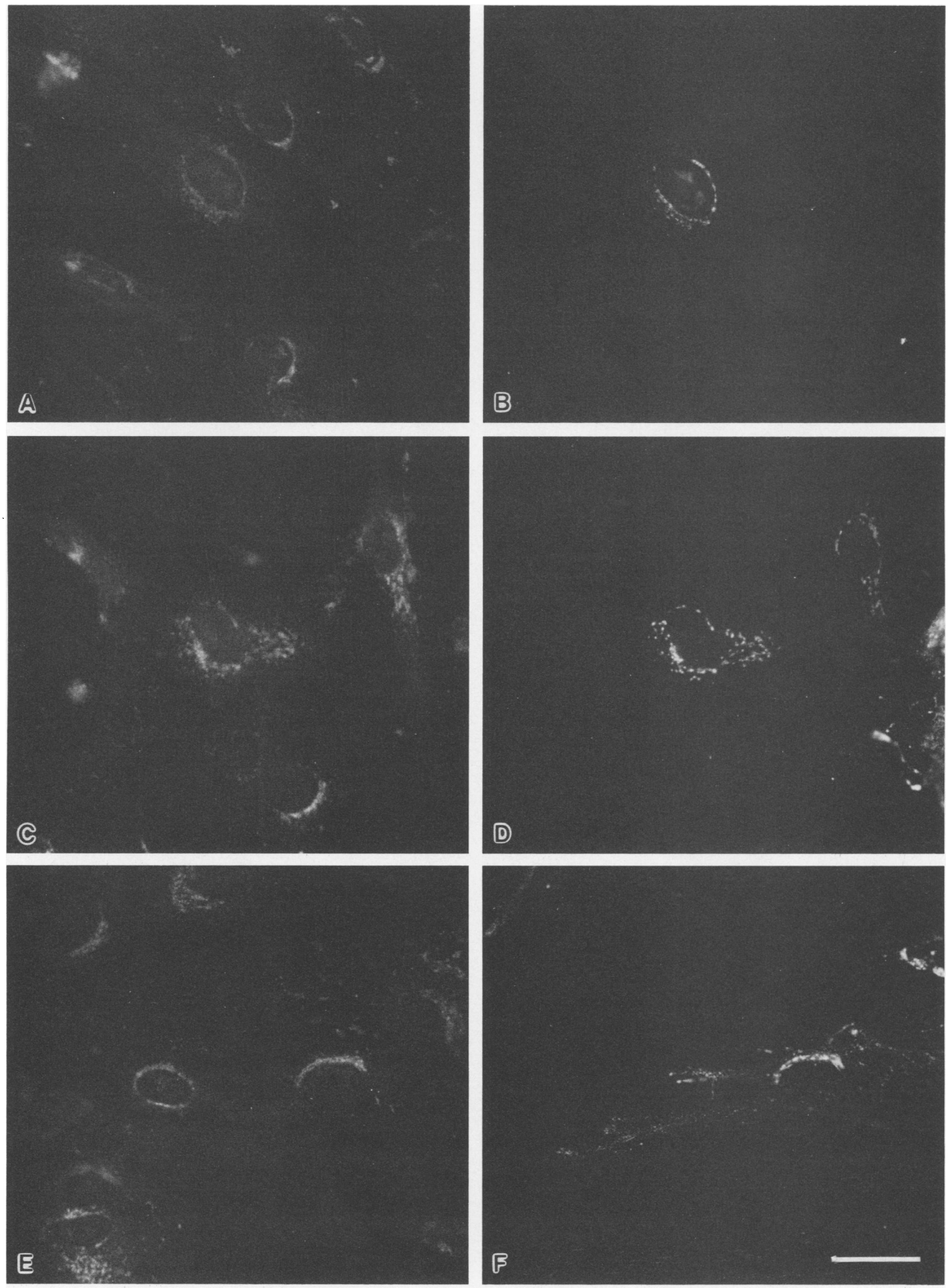
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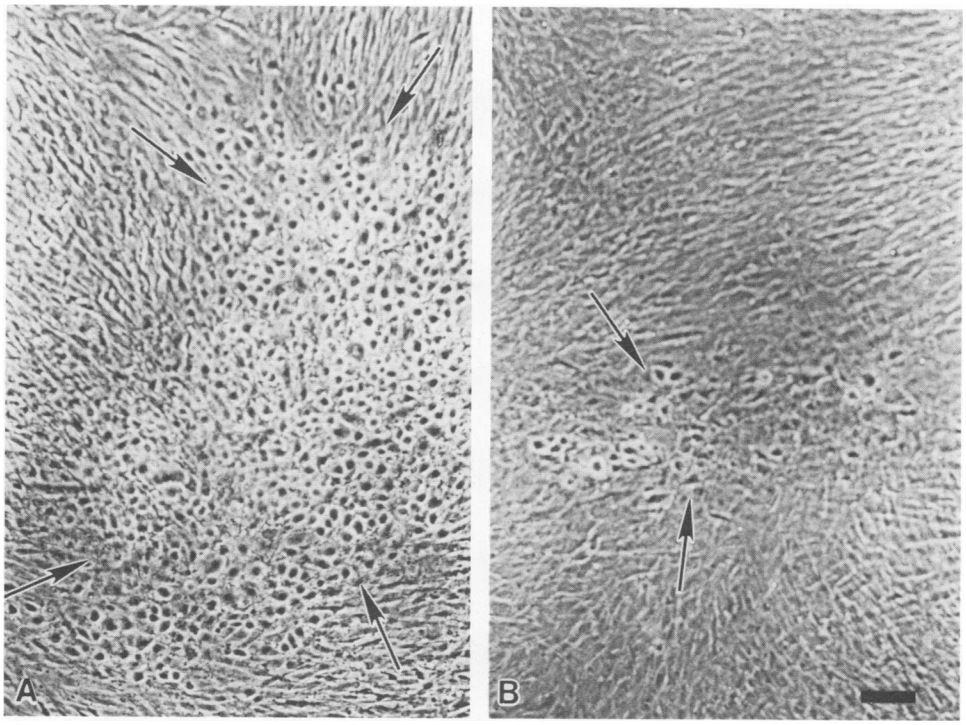

FIG. 8. Man 6-P inhibits VZV-induced CPE. Monolayers of HELFs were infected with cell-free VZV in the absence (A) and presence (B) of 20 mM Man 6-P; 3 days later the monolayers were examined and photographed. In the absence of the phosphorylated monosaccharide, larger plaques of infected cells were detected (arrows). The infected cells became more round in contour and refractile. The Man 6-P-treated monolayer was free of the large virus-induced plaques. (B) In some instances, the Man 6-P-treated monolayers contained small regions of altered morphology (arrows), suggestive of a small plaque. Bar, 100 μm.

protected HELFs from the CPEs of the virus. Additional cultures were exposed to chloroquine (50 μM) for 4 h, washed, and then incubated in the absence of the weak base for 1 and 24 h before being challenged with cell-free VZV. Virus-induced CPE was observed in the cultures corresponding to 22 and 63% of that observed in non-chloroquine-treated monolayers after 1 and 24 h of chase, respectively. Viral infectivity, therefore, was blocked reversibly by chloroquine.

DISCUSSION

The strict cell association of VZV when grown in culture contrasts with the behavior of HSV-1, which is secreted by infected cells into the surrounding medium (5, 14). Since the glycoproteins associated with secreted virions contain complex-type asparagine-linked oligosaccharides (4), the intracellular maturation of the viral particles appears to proceed along the same pathway as that followed by secretory glycoproteins in uninfected cells. In general, secreted glycoproteins are synthesized in the rough ER in which they are cotranslationally glycosylated by the addition of preformed high-mannose-type oligosaccharides (25). The glycoproteins subsequently migrate to the Golgi apparatus in which the high-mannose-type oligosaccharides are converted to complex-type units, and ultimately they are discharged via exocytosis (22). The VZV-encoded glycoproteins must similarily pass through the Golgi apparatus since many of their high-mannose oligosaccharides are converted to complex-type units. In the present study, total cell-associated viral glycoproteins were analyzed. While it is possible that glycoproteins which do not assemble into a virion behave differently from virion-associated species, this and previous electron microscopic analyses of HSV and VZV-infected cells detected intact virions in the Golgi apparatus (12, 34). We assume, therefore, that the nucleocapsids become enveloped, because they bud from the inner nuclear envelope and enter the ER cisterna, and that both capsid-associated and nonassociated forms of the viral glycoproteins are processed comparably as they move through the secretory apparatus of the host cell. An alternative mechanism of virion envelopment, however, has been proposed in which the glycoproteins associate with the nucleocapsid in a post-Golgi process (20).

After passage through the Golgi apparatus, VZV is diverted from the secretory pathway to accumulate within large cytoplasmic vacuoles (5). This site of divergence is comparable to the point at which newly synthesized acid hydrolases are withdrawn from the secretory pathway (24). Acid hydrolases bind to specific Golgi-associated Man 6-P receptor proteins and subsequently are shuttled to a prely-

FIG. 7. Colocalization of Man 6-P receptor and gpI immunoreactivities in VZV-infected HELFs. Monolayers of HELFs were infected with VZV. At 24 h postinfection, the monolayer was fixed, made permeable, and processed for immunodetection of the Man 6-P receptor and gpI. Each pair of micrographs represents the same fields photographed to show the immunofluorescence of the Man 6-P receptor (panels A, C, and E) and gpI (panels B, D, and F). Surface Man 6-P receptor immunoreactivity is apparent in panel B. Bar, 50 μm.

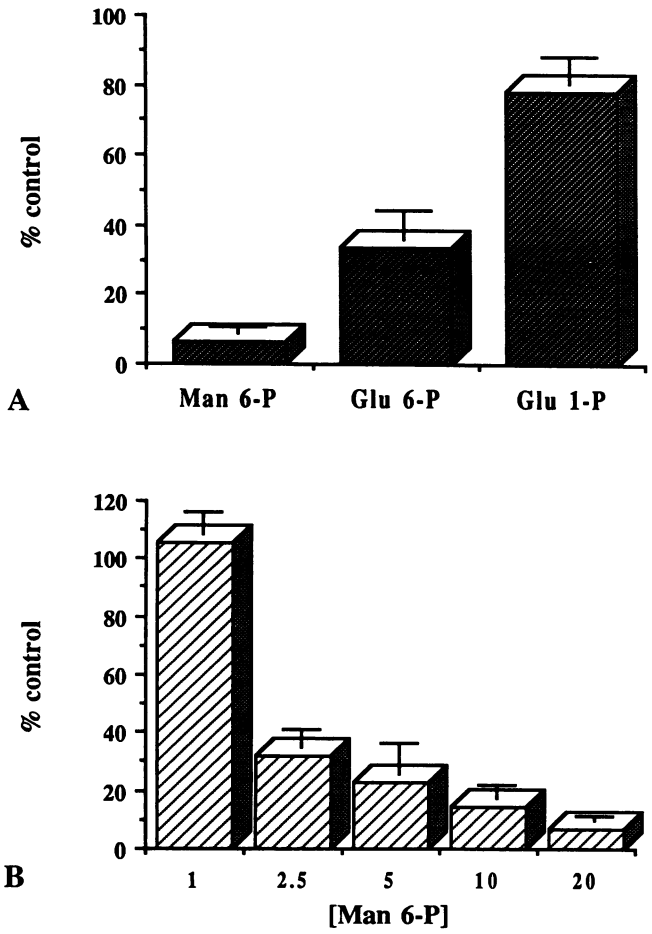

FIG. 9. Man 6-P inhibits VZV-induced CPE. (A) HELF monolayers were incubated with cell-free VZV in the presence of 20 mM Man 6-P, Glu 6-P, or Glu 1-P. After a 45-min adsorption, the cells were cultured for 2 days, trypsinized, and passaged to fresh monolayers; 2 days later, the number of infectious centers was determined. The level of CPE is expressed as a percentage of that observed in the absence of any phosphorylated monosaccharide. (B) HELF monolayers were incubated with cell-free VZV and the indicated concentration of Man 6-P. After the 4-day incubation period, the level of CPE was determined and is expressed as a percentage of that observed in the absence of the monosaccharide. Data from four separate experiments are included.

sosomal compartment (2, 16). By analogy to the targeting of lysosomal enzymes, withdrawal of VZV from the secretory pathway would be expected to require an active recognition event. Two observations suggest that this recognition may occur via the Man 6-P receptor. First, Man 6-P protects HELFs from the CPE of VZV in a concentration-dependent manner. The rank order of potency of Man 6-P, Glu 6-P, and Glu 1-P in inhibiting VZV infection of HELFs is the same as the rank order of the monosaccharides for inhibiting the internalization of acid hydrolases via the Man 6-P receptor (21). It is thus possible that the Man 6-P receptor is required for entry of the virus. Since weak bases such as chloroquine alkalinize intracellular compartments and thereby prevent dissociation of the Man 6-P receptor and its ligand (15), the reversible protection offered by chloroquine against VZV infectivity is consistent with a role for this receptor. At present, direct evidence that Man 6-P or chloroquine prevent entry of the virus is lacking. Second, asparagine-linked oligosaccharides attached to newly synthesized VZV glycoproteins contain phosphorylated mannose residues that comigrate with Man 6-P by paper chromatography. The viral glycoprotein-associated phosphorylated oligosaccharides are not comparable to the high-mannose-type units found on acid hydrolases. The viral oligosaccharides produced at 20°C (i) contain mannose and mannose phosphate in a 2 to 1 ratio, (ii) are resistant to alkaline hydrolysis, (iii) are released by glycopeptidase F, and (iv) do not interact with ConA-Sepharose. These properties suggest that the phosphorylated units are tri- and/or tetraantennary-type oligosaccharides in which one of the three core mannose residues of the complex-type oligosaccharide is phosphorylated. Precedent for this type of structure exists, since thyroglobulin contains phosphorylated complex-type oligosaccharides (19).

If an interaction between newly synthesized VZV virions and the Golgi-associated Man 6-P receptor is responsible for the diversion of newly assembled virions from the secretory pathway, then phosphorylation should occur before the exit of virions from the Golgi apparatus. When pulse-labeled VZV-infected HELFs were chased at 20°C, the glycoproteins were phosphorylated. Migration of newly synthesized glycoproteins from the ER to the Golgi is slowed and exit from the Golgi is disrupted at 20°C (37, 38). The [^{3}H]mannose-labeled VZV-encoded glycoproteins remained predominantly in their precursor forms after 3 h of chase at 20°C, as evidenced by the persistence of the pgpI 77-kDa species. Mature gpI migrates more slowly during gel electrophoresis (molecular size of 96 kDa), and the increase in its apparent molecular size coincides with the acquisition of complex-type oligosaccharides (30). The pulse-labeled pgpI molecules, therefore, apparently did not traverse the entire Golgi apparatus at 20°C and, as a result, contained predominantly high-mannose-type oligosaccharides. The few complex-type glycopeptides recovered from the 77-kDa species, however, contained mannose phosphate residues, indicating that phosphorylation of the viral glycoprotein oligosaccharides occurred before completion of maturation of the proteins.

The CI Man 6-P receptor cycles between the cell surface, the Golgi apparatus, and endosomes (9, 13, 24). Within VZV-infected HELFs, the major intracellular location of the receptor corresponded to the site of viral glycoprotein gpI accumulation. This colocalization is consistent with the proposed interaction between the receptor and the newly synthesized virions. Many of the cytoplasmic structures that contained virions were not accessible to endocytosed HRP. Components of the *trans*-Golgi reticulum do not accumulate this fluid-phase tracer (17); therefore, the absence of HRP as well as the perinuclear location of the structures is consistent with the idea that virion-containing vesicles are derived from elements of the Golgi apparatus. Some of the virion-containing vesicles, however, accumulated tracer when the infected cells were exposed briefly to HRP. These vesicles must be components of the endosomal compartment. Our inability to detect virions within lysosomes may indicate that the viral structures did not proceed beyond endosomes to lysosomes or that the virions were degraded within lysosomes and were not discernable as a result of their altered morphology.

Virions within the cytoplasmic vacuoles of VZV-infected HELFs possessed a pleomorphic appearance as has been observed previously in other cell types (5). The interiors of the *trans*-Golgi reticulum and endosomal compartments possess a low pH and contain newly synthesized acid hydrolases which are in transit to lysosomes (1, 13, 44). It is

possible that the low pH and hydrolytic enzymes degrade the enclosed virions and lead to their pleomorphic appearance; thus, the proposed interaction between the host cell Man 6-P receptor and the phosphorylated oligosaccharides of the virion glycoproteins accounts for several of the unusual aspects of VZV behavior in culture.

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