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Three Major Glycoprotein Genes of Varicella-Zoster Virus Whose Products Have Neutralization Epitopes

PAUL MALCOLM KELLER, BEVERLY J. NEFF, AND RONALD W. ELLIS*

Virus and Cell Biology Research, Merck Sharp & Dohme Research Laboratories, West Point, Pennsylvania 19486

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Varicella-zoster virus (VZV) codes for approximately eight glycosylated polypeptides in infected cell cultures and in virions. To determine the number of serologically distinct glycoprotein gene products encoded by VZV, we have developed murine monoclonal antibodies to purified virions. Of 10 monoclonal antibodies which can immunoprecipitate intracellular VZV antigens and virion glycoproteins, 1 (termed gA) reacted with gp105, 1 (termed gB) reacted with gp115 (intracellular only), gp62, and gp57, and 8 (termed gC) reacted with gp92, gp83, gp52, and gp45. The anti-gA monoclonal antibody neutralized VZV infectivity in the absence of complement. All eight anti-gC monoclonal antibodies neutralized only in the presence of complement. An anti-gB monoclonal antibody obtained from another laboratory also neutralizes in the absence of complement. Since the above reactivities account for all major detectable VZV glycoprotein species, the data strongly suggest that VZV has three major glycoprotein genes which encode glycosylated polypeptides with neutralization epitopes.

Varicella-zoster virus (VZV) is a human herpesvirus which causes chickenpox (varicella) as a result of primary infection and shingles (zoster) after reactivation of latent virus. The immune response to VZV plays a critical role in the prevention of primary infection, since attenuated VZV strains can confer protective immunity (1, 2, 6). Due in part to its highly cell-associated nature, VZV has not been as well characterized at a molecular level as the herpes simplex viruses. In infected cells, the virus specifies ca. 30 species of polypeptides ranging in molecular weight from 16,000 to ca. 240,000 (15, 25, 26, 30), of which 6 to 8 are glycoproteins (12, 25, 26, 31). The immune response after primary and recurrent VZV infections has been characterized: ca. 16 antigens, including three to four major glycoproteins, can induce antibodies (13, 30, 31). However, those antigens critical to inducing protection against primary infection or reactivation are not well resolved. Recently, several laboratories reported the production of monoclonal antibodies against VZV virions (10, 14, 20). Differences in nomenclature have left unclear the actual number and type of glycoprotein antigens in VZV as well as those recognized by these monoclonal antibodies or polyclonal antisera. The objective of this investigation was to characterize those polypeptides which induce neutralizing antibodies by producing a complete set of monoclonal antibodies against all of the major glycoproteins. We have used these antibodies to identify and characterize reactive antigens and to approach the question of how many distinct viral genes might specify such antigens.

Ten-week-old BALB/c mice were immunized subcutaneously at multiple sites with 20 μg (measured as in reference 17) of purified virus (VZV strain KMcC [18]) emulsified in complete Freund adjuvant, followed 2 weeks later by intraperitoneal boosting with 25 μg of purified VZV without adjuvant. All sera assayed 2 weeks after the second injection had antivirus titers of >400 and anticell titers of <50 in the live cell membrane fluorescence (FAMA) assay and reacted with VZV proteins in Western blots of infected cell extracts (data not shown). One month after the second immunization, mice were injected intravenously with 25 μg of purified VZV without adjuvant. Three days later, spleens were removed from the two mice with the highest anti-VZV titers. Spleen cells were fused with SP2/O mouse myeloma cells (Institute for Medical Research), and hybridoma cells were plated and cultured by previously published procedures (19). Two weeks later, supernatant fluids from wells of actively growing cells were tested for anti-VZV reactivity by the FAMA assay. Cells secreting antibodies reactive with VZV membrane antigens were cloned by limiting dilution and expanded into ascites fluids. In general, two monoclones from each polyclone were used to produce ascites fluids. No differences in the specificities of any two "sister" clones were observed.

Ten independent monoclonal antibodies in ascites fluids were produced which recognized VZV membrane antigens. When tested for reactivity to VZV-infected MRC-5 cells, FAMA titers ranged from 1:32,000 to 1:256,000, whereas none of the antibodies reacted by FAMA to uninfected cells (Table 1). Antibody subclasses were characterized (Table 1). The antibody concentration in each ascites fluid was ca. 2 mg/ml.

The serological specificities of the monoclonal antibodies were analyzed by immunoprecipitation. Antibodies, as well as control ascites fluid, were reacted with [^{35}S]methionine-labeled infected cell extracts, [^{14}C]glucosamine-labeled infected cell extracts, and [^{35}S]methionine-labeled purified virions. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis profiles are presented (Fig. 1A to C), and the interpretations of these profiles are summarized (Table 1). We observed that monoclonal antibodies C1 to C8 displayed virtually identical reactivities to VZV polypeptides (with the exceptions noted in footnote *f* of Table 1); thus, the profile displayed with antibody C8 (Fig. 1A2, B2, and C2) is representative of the C1 to C8 group. Three patterns of reactivity among the 10 monoclonal antibodies were observed. (i) Clone A1 antibodies recognized a single polypeptide termed gp105 (105,000-molecular-weight glycoprotein) from both infected cells and virions. (ii) Clone B1 antibodies recognized p110 (nonglycosylated), gp115, gp62, and gp57 from infected cells and only gp62 and gp57 from the virion. (iii) The eight group C antibodies recognized gp45 from

* Corresponding author.

TABLE 1. Specificity of the reactivities of monoclonal antibodies

Clone[a]	Antibody[b] isotype	Immunoprecipitation[c]		Western blot[c]		Antigen group recognized	Neutralization titer[d]		FAMA titer[e]
		Infected cell extracts	Purified virions	Infected cell extracts	Purified virions		C′ present	C′ absent	
A1	IgG2a	gp105	gp105	None	None	gA	1:32,000	1:32,000	1:32,000
B1	IgG1	p110, gp115, gp62, gp57	gp62, gp57	None	None	gB	<1:100	<1:100	1:64,000
C1	IgG1	gp92, gp83, gp52, gp45	gp92, gp83, gp52	gp92, gp83, gp52, gp45	gp92, gp83, gp52	gC	1:4,000	<1:100	1:256,000
C2	IgG1						1:8,000	<1:100	1:64,000
C3[f]	IgG2a						1:16,000	<1:100	1:256,000
C4	IgG1						1:8,000	<1:100	1:128,000
C5[f]	IgG1						1:4,000	<1.100	1:32,000
C6	IgG2a						1:4,000	<1:100	1:64,000
C7[g]	IgG2b						1:8,000	<1:100	1:256,000
C8	IgG1						1:16,000	<1:100	1:64,000

[a] Hybridoma cells were cloned by limiting dilution onto irradiated (10,000 rads from a ^{60}Co source) MRC-5 cells in 96-well plates. For production of antibody in ascites fluid, mice primed with pristane (2,6,10,14,-tetramethylpentadecane) were injected with 4×10^6 cells per mouse. Approximately 10 days later, ascites fluid was collected.

[b] Immunoglobulin subclass was determined by immunodiffusion assay against goat monospecific antisera to mouse immunoglobulin G1 (IgG1), IgG2a, IgG2b, and IgG3 (Research Products International Corp.).

[c] Antibodies in ascites fluids were tested at 1:250. These columns summarize the data of Fig. 1 and 2.

[d] The procedure followed was a modification of the plaque reduction test (3). Serially diluted ascites fluids were mixed with an equal volume of cell-free VZV strain KMcC, either with or without 10% fresh guinea pig complement (C′) and incubated for 1 h at room temperature. The test was set up such that 25 to 50 PFU was added per well, and four wells were assayed per antibody dilution. The antibody-virus mixture (0.1 ml) was inoculated onto the drained surface of Vero cells in 24-well (16-mm) plates. The virus was allowed to adsorb for 1 h at 36°C, and the cells were refed and incubated for 7 days. The antibody titer was calculated as the reciprocal of the dilution which reduced the number of plaques by 50%.

[e] The FAMA test was performed essentially as previously described (11, 29), utilizing VZV-infected or uninfected FS-4 (human foreskin fibroblast) as indicator cells and fluorescein-labeled goat anti-mouse immunoglobulin (gamma and light chain specific; TAGO Inc.) as a second antibody. The antibody titer was calculated as the reciprocal of the dilution which gave detectable immunofluorescence.

[f] These clones demonstrated a preference in serological reactivity for gp92 over gp83.

[g] This antibody was nonreactive on Western blots.

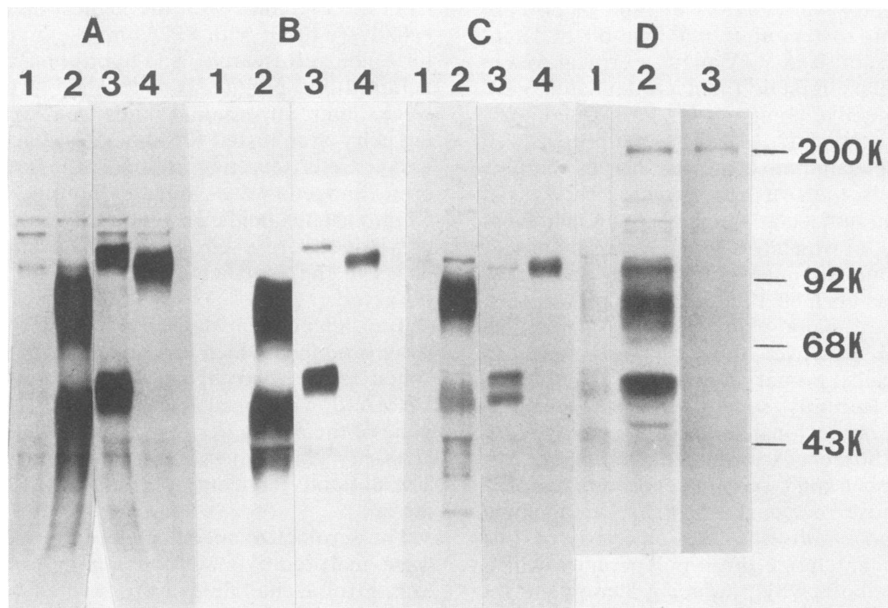

FIG. 1. Electrophoretic analysis of immunoprecipitations of VZV polypeptides. Human diploid fibroblast (MRC-5) cells were grown at 36°C in Eagle basal metabolic medium with Earle salts and 10% fetal calf serum and were used as a virus host between passages 25 and 30 for the KMcC strain of VZV (18), which was passaged as a cell-associated stock. VZV-infected cells showing 50% cytopathic effect in 850-cm^2 roller bottles were labeled either with 10 ml of methionine-free Dulbecco modified Eagle medium plus 2% dialyzed fetal calf serum and 50 μCi of L-[^{35}S]methionine per ml (1,450 Ci/mmol; Amersham Corp.) or with 10 ml of Dulbecco modified Eagle medium containing 10% of the normal glucose concentration, 2% dialyzed fetal calf serum, and 10 μCi of D-[^{14}C]glucosamine hydrochloride per ml (54 Ci/mmol; Amersham Corp.). After 24 h, sonicated cell extracts were prepared by washing cell monolayers twice with 0.04 M sodium phosphate–0.15 M sodium chloride (phosphate-buffered saline), and 3 ml of SPG buffer (0.04 M sodium phosphate, 5% sucrose, 0.1% sodium glutamate) was added to each roller bottle. Cells were scraped into a centrifuge tube on ice, sonicated three times for 60 s each, and centrifuged at 2,000 × *g* for 10 min at 4°C. Sonicated cell extracts were stored at −70°C, unless used for virus purification, in which case they were processed immediately as follows. Extracts were layered onto 10 to 35% sucrose density gradients (in SPG buffer) and centrifuged for 25 min at 104,000 × *g* at 4°C. The lower virus band was removed from the side of the tube with a syringe, layered on a 35 to 55% sucrose density gradient (in SPG buffer), and centrifuged at 131,000 × *g* for 8 h at 4°C. The lower band was collected, diluted with SPG buffer, and rebanded on a 35 to 55% sucrose density gradient. The virus band then was collected, diluted in SPG buffer, pelleted at 131,000 × *g* for 2 h at 4°C, resuspended in SPG buffer, and stored at −70°C until needed. Electron microscopy of the purified material, performed by B. Wolanski, showed 80% enveloped virions. After purification of differentially labeled infected (^{3}H-labeled) and uninfected (^{14}C-labeled) cell lysates, the virus preparation was estimated to be purified 35-fold. Immunoprecipitation analyses, sample preparations, and SDS-polyacrylamide gel electrophoresis were performed as described in the legend to Fig. 3. Antigens used in these analyses were (A) [^{35}S]methionine-labeled sonicated infected cell extracts, (B) [^{14}C]glucosamine-labeled sonicated infected cell extracts, or (C) [^{35}S]methionine-labeled purified virions. These samples were immunoprecipitated by the following monoclonal antibodies (lanes): 1, control ascites fluid; 2, C8; 3, B1; 4, A1. (D) Electrophoretic analysis of the major glycoprotein species of VZV; lanes: 1, lysate of purified [^{14}C]glucosamine-labeled virions; 2 and 3, [^{14}C]glucosamine-labeled sonicated cell extracts immunoprecipitated by (lane 2) convalescent zoster antiserum (VZV FAMA titer, 1:800) or (lane 3) normal human serum (VZV FAMA titer, <1:10). Molecular weight markers are myosin (200,000 [200K]), phosphorylase *b* (92K), bovine serum albumin (68K), and ovalbumin (43K).

infected cells only and gp92, gp83, and gp52 from both infected cells and virion.

To characterize further the specificities of the monoclonal antibodies, both purified virus and infected cell extracts were denatured and resolved on Western blots, and ascites fluids were tested for reactivity. Neither A1 nor B1 reacted with any polypeptide, whereas seven of the eight group C antibodies reacted with the entire group of cellular antigens, gp92, gp83, gp52, and gp45. From purified virions, gp92 and gp83 predominated in reactivity, as was the case in immunoprecipitation analyses (Fig. 1C2). Representative profiles with one of the seven reactive group C antibodies are shown (Fig. 2). Significantly, the Western blot data reenforce the grouping of various polypeptides, i.e., gp92, gp83, gp52, and gp45, to a single family of antigens by ruling out possible nonspecific coprecipitations. It is noteworthy that all seven group C antibodies reactive in Western blots also were positive by immunoprecipitation. This is in contrast to an earlier study, in which Western blot screening alone actually detected a greater number of monoclonal antibodies than did immunoprecipitation screening alone (5).

Several of the polypeptides defined by immunoprecipitation analysis exhibited similar electrophoretic mobilities. To investigate further the antigenic distinctions and possible serological groupings among viral gene products, cross-clearing immunoprecipitations were performed among the monoclonal antibodies with [^{35}S]methionine-labeled infected cell extracts (Fig. 3). After two clearings of the extract with A1, antibody A1 did not immunoprecipitate any additional gp105 (Fig. 3A3), whereas antibody B1 immunoprecipitated gp115, p110, and gp62 efficiently from the A1-cleared extract (Fig. 3B3). The converse experiment, with antibody B1 followed by A1 (lane groups C and D), as well as with antibody C6 (lane groups E and F), demonstrates similar distinctions. Therefore, our monoclonal antibodies define three families of antigenically distinct glycoproteins, which we term gA, gB, and gC.

A cursory comparison between the glycoprotein species resolved above and those reported elsewhere (12, 13, 25, 26, 31) suggested that our monoclonal antibodies in toto had detected all major VZV glycoproteins. To address this question directly, nonimmunoprecipitated [^{14}C]glucosamine-labeled virions were lysed and electrophoresed (Fig. 1D1). Also, [^{14}C]glucosamine-labeled infected cell extracts were immunoprecipitated with a high-titer human convalescent zoster serum (Fig. 1D2). The major detectable glycoprotein species in these two experiments approximately coincided with the sum of the gA, gB, and gC bands detected by the monoclonal antibodies. The existence of other minor glycoprotein bands is not ruled out by this analysis. Nevertheless, our analysis with monoclonal antibodies strongly suggests that gA, gB, and gC are the three major VZV glycoprotein genes.

Monoclonal antibodies were tested for biological activity against VZV by the neutralization assay (Table 1). Clone A1 antibodies neutralized strongly in the absence of complement. The eight monoclonal antibodies reactive with gC polypeptides neutralized only in the presence of complement. Our gB-reactive antibody failed to neutralize. However, we obtained a monoclonal antibody from C. Edson for which we could demonstrate serological reactivity in immunoprecipitations identical to clone B1 antibodies (data not shown; reactive with gp63, gp125 in the nomenclature of Edson et al. [C. Edson, submitted for publication]). These antibodies neutralize infectivity in the absence of complement (data not shown; Edson, submitted for publication).

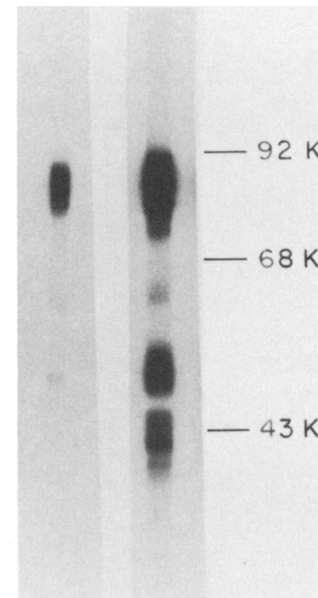

FIG. 2. Western blot analysis of the reactivity of monoclonal antibodies to VZV polypeptides. After sample preparation, as described in the legend to Fig. 3, polypeptides were electrophoresed in 7.5% discontinuous SDS-polyacrylamide gels under reducing conditions (16) and transferred to nitrocellulose by the method of Burnette (7). All subsequent incubations were performed at room temperature. After transfer, the nitrocellulose was immersed in buffer A (0.15 M NaCl, 50 mM Tris [pH 7.6], 0.1% NaN_3) plus 20% agammaglobulin in calf serum overnight, washed with incubation buffer (5% agammaglobulin calf serum in buffer A), and placed in antisera of the desired dilution in incubation buffer for 2 h with rocking. The nitrocellulose then was washed once with buffer A for 10 min, twice with buffer A plus 0.05% Triton X-100 for 10 min, and once with buffer A again. When mouse antibodies were being used, this step was followed by incubation with a 1:1000 dilution of rabbit anti-mouse immunoglobulin G heavy and light chains in incubation buffer for 1 h, followed by the above washing procedure. The blot then was incubated with 0.25 μCi of ^{125}I-protein A (New England Nuclear Corp.) per ml in incubation buffer for 1 h, washed as above, dried, and autoradiographed. Purified virions (lane 1) or sonicated cell lysates (lane 2) were electrophoresed, blotted, and reacted with monoclonal antibody C5.

Therefore, all three major VZV glycoprotein genes encode polypeptides with neutralization epitopes. This result is not unlike that found in the herpes simplex virus system, in which four distinct glycoprotein genes encode glycopeptides with neutralization epitopes (4, 21, 23, 27).

The differential representations of various glycoproteins among labeled cell extracts and virions enable us to suggest certain hypotheses about the relationships of the different polypeptide species. (i) The gA gene product gp105 probably corresponds to gp115 (gpl) of Shiraki et al. (26) and to gp118 of Grose et al. (14), who report that a monoclonal antibody to gp118 also neutralizes in the absence of complement. (ii) The gB gene products gp62 and gp57 probably represent the final processed forms of gB in the virion, since gp115 and p110 are present only in infected cells. Furthermore, the nonglycosylated p110 might represent a precursor to gp115. Similar conclusions have been reached by others (Edson, submitted for publication; gp116, gp106, and gp64 in reference 20), although each group detected only a single virion glycoprotein species of molecular weight 63,000 to 64,000 and one group detected two higher-molecular-weight glycoproteins (20). Similar pulse-chase relationships have been

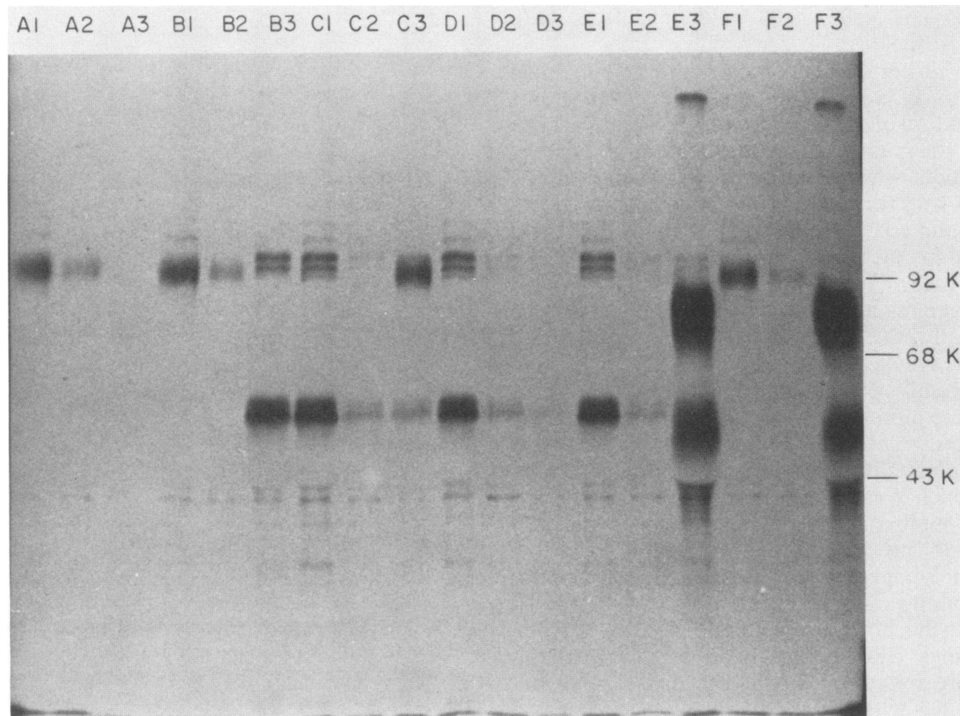

FIG. 3. Antigenic distinctiveness among VZV glycoproteins as demonstrated by cross-clearing immunoprecipitations. Formalin-fixed staph A cells (Bethesda Research Laboratories) were washed as described by Richert et al. (24), resuspended in BW buffer (0.1% SDS, 0.5% deoxycholate, 1% Triton-X 100, 0.01 M Tris [pH 8.0], 0.1 M NaCl, 0.002 M EDTA) at 6% (vol/vol) and stored at −70°C for immunoprecipitation of human antibodies. For immunoprecipitations with mouse antibodies, the staph A preparation was pelleted, rabbit anti-mouse immunoglobulin G heavy and light chains (Cappel Laboratories) were added, and the suspension was brought back to the original volume with phosphate-buffered saline. After the suspension was mixed for 2 h at room temperature, it was washed twice with phosphate-buffered saline brought back to 6% (vol/vol) in 0.04 M Tris (pH 8.0)–0.02% NaN_3, and stored at 4°C for up to 1 month before use. Radiolabeled sonicated cell extracts or purified virions were brought to 1% Triton-X 100 and 0.5% deoxycholate and reclarified by centrifugation at 12,000 × *g* for 20 min at 4°C. Antigen fractions containing ca. 10^6 cpm were brought to a volume of 300 μl with 0.02 M Tris-hydrochloride (pH 7.4)–0.1 M NaCl–1% Triton-X 100–0.5% deoxycholate–0.005 M $MgCl_2$ and various volumes of ascites fluids (1 to 10 μl). The mixture was incubated for 2 h at 4°C, and 50 μl of the staph A preparation was added for a 1-h incubation at 4°C with frequent mixing. The mixture then was pelleted by centrifugation at 18,000 × *g* for 3 min at 4°C, and the pellet was washed four times in 1 ml of BW buffer. After the last wash, the antigen was eluted by the addition of 40 μl of 2% SDS–4% β-mercaptoethanol–10% glycerol–0.063 M Tris phosphate (pH 7.5) and boiling for 3 min. Clearing immunoprecipitations were performed as described above, except that the antibody-treated supernatants from the first antigen-antibody-staph A reaction were saved and mixed with a second antibody for the second and third rounds of immunoprecipitation. Precipitated proteins were eluted in each cycle from the staph A as described above. Samples were electrophoresed in 7.5% discontinuous SDS-polyacrylamide gels under reducing conditions (16). When immunoprecipitations were performed, the gels were fixed in 7% acetic acid–25% methanol overnight, washed three times with 100 ml of dimethyl sulfoxide for 15 min, and impregnated with 2′,5′-diphenyloxazole in dimethyl sulfoxide (21 g/100 ml) for 1 h. After the gels were washed in cold running water, they were dried in vacuo and fluorographed. [^{35}S]methionine-labeled infected cell extracts were immunoprecipitated sequentially with monoclonal antibodies up to three times. Each triplet of lanes (A through F) represents a single sequential experiment with the first (1), second (2), and third (3) monoclonal, respectively. Monoclonal antibodies used for each lane were: (A) 1, A1; 2, A1 and A1; 3, A1, A1, and A1; (B) 1, A1; 2, A1 and A1; 3, A1, A1, and B1; (C) 1, B1; 2, B1 and B1; 3, B1, B1, and A1; (D) 1, B1; 2, B1 and B1; 3, B1, B1, and B1; (E) 1, B1; 2, B1 and B1; 3, B1, B1, and C5; (F) 1, A1; 2, A1 and A1; 3, A1, A1, and C5.

found in herpes simplex virus type 2 (4, 21). (iii) The gC gene products gp92, gp83, gp52, and gp45 represent the predominant viral glycoprotein species based on SDS-polyacrylamide gel electrophoresis of purified [^{14}C]glucosamine-labeled virions as well as on immunoprecipitations with convalescent zoster sera. They appear to be the most immunogenic glycoproteins based upon the ease of isolation of monoclonal antibodies to gC, 8 of 10 in this report, 4 of 5 in another report (gp98 and gp63 [14]), and 8 of 12 in another report (gp94, gp83, gp55, and gp45 [20]). The gp92 and gp83 species seem to represent the mature virion polypeptides, as deduced by their overrepresentation relative to gp55 in virions. It is noteworthy that monoclonal antibodies to gC detect more than one glycoprotein, an observation that has been made in other members of the herpesvirus family, e.g., herpes simplex virus (4, 8, 23), Epstein-Barr virus (9, 28), and cytomegalovirus (22). Since these cross-reactions do not appear to be nonspecific in nature, the multiple polypeptide forms could be processed from related but distinct precursors, could share pulse-chase relationships, or could represent heterogeneities in glycosylation patterns. We have been able to use our anti-gC antibodies to genetically map the VZV gC gene (Ellis et al., submitted for publication).

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