The Glycoprotein Products of Varicella-Zoster Virus Gene 14 and Their Defective Accumulation in a Vaccine Strain (Oka)

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Many characteristics of the putative protein encoded by varicella-zoster virus (VZV) open reading frame (ORF) 14 indicate that it is a glycoprotein, which has been designated gpV. To identify the protein products of the gene, the coding sequences were placed under the control of the vaccinia virus p7.5 promoter and recombinant vaccinia viruses were constructed. Heterogeneous polypeptides with molecular weights of 95,000 to 105,000 (95K to 105K polypeptides) were expressed in cells infected by a vaccinia virus recombinant (vKIP5) containing ORF 14 from VZV Scott but were not expressed by control vaccinia viruses. These polypeptides were recognized by antibodies present in human sera that contained high levels of anti-VZV antibodies. Conversely, antisera raised in rabbits inoculated with vKIP5 reacted specifically with heterogeneous 95K to 105K polypeptides present in VZV Scott-infected but not uninfected cells; these polypeptides show a patchy plasma membrane fluorescence pattern in VZV Scott-infected cells. These same antisera neutralized VZV strain Scott infectivity in the absence of complement. Endoglycosidase F treatment of isolated gpV polypeptides and tunicamycin treatment of cells infected with the vKIP5 recombinant indicated that the polypeptides were glycosylated. Three sets of data imply that the VZV strain Oka, which has been used to produce a live attenuated virus vaccine, accumulates low levels of gpV polypeptides relative to wild-type strains: (i) blocking of antibodies in human sera with excess VZV Oka-infected cell antigen yielded residual antibodies which were reactive with the 95K to 105K gpV polypeptides expressed in cells infected by VZV strain Scott and by the vKIP5 vaccinia virus recombinant, but not with Oka-infected cell polypeptides; (ii) antisera raised to vKIP5 detected very low levels of reactive polypeptides made in VZV Oka-infected cells and neutralized VZV Oka virus much less efficiently than VZV Scott; and (iii) comparisons of the reactivity of sera from live attenuated virus vaccinees with sera derived from patients recovering from wild-type infections indicated greatly reduced levels of gpV-specific antibodies in some vaccinees.

Varicella-zoster virus (VZV) is the causative agent of two clinical manifestations: varicella, or chickenpox, which is usually contracted in childhood, and zoster, or shingles, which is a painful disease caused by reactivation of the latent virus. Zoster is of growing significance, occurring more frequently as the elderly population increases. Immunocompromised individuals, particularly susceptible children, suffer higher morbidity and some mortality from both diseases. This spurred the development of a live attenuated virus vaccine (LAVV) aimed at prevention of varicella in leukemic and immunosuppressed children (34). LAVV was derived by passage of a virus isolate (strain Oka) through semipermissive and permissive tissue cultures. It is currently undergoing extensive clinical trials throughout the world (15) and might be given to children as a routine vaccination in early childhood, possibly in conjunction with the measles, mumps, and rubella vaccines (1). The molecular basis for its attenuation is not known.

The identification of VZV-specified glycoproteins is of primary importance in studies of the virus and of virus vaccines, since they are likely to be among the primary targets for both humoral and cellular immune responses in the infected individual. Analysis of the sequence of the VZV genome has revealed at least five potential glycoprotein genes (open reading frames [ORFs] 68, 31, 37, 67, and 14),

whose products are designated gpI, gpII, gpIII, gpIV, and gpV (4, 5, 21). These have been defined on the basis of typical glycoprotein structural characteristics predicted from ORFs within VZV DNA (10, 19, 21). Initial characterization of polypeptides synthesized in cells infected with VZV revealed a large number of electrophoretically heterogeneous glycoprotein species. Monoclonal antibodies which react with subsets of these virus-specified glycoproteins have been isolated, and their use has shown that many of the species are related by being either alternately processed forms of the same polypeptide backbone (12, 13, 16, 20, 26–28) or are multiple forms of the same polypeptide linked by disulfide bonds (e.g., gpII; 16, 36). Collectively, these experiments allowed the identification of glycoproteins expressed by genes encoding VZV gpI (ORF 68), gpII (ORF 31), and gpIII (ORF 37) (10–13, 16, 20, 28, 37). The products of VZV ORF 67 (gpIV) have also been identified recently by using antibodies raised to a synthetic peptide derived from part of the predicted amino acid sequence of the ORF (6) and by a cross-reactive site present on both gpI and gpIV (35). GpI, gpII, and gpIII have all been shown to possess epitopes which are targets for neutralizing antibodies, and complement appears to be required for neutralization by anti-gpI antisera (11–13, 20, 37). Antibodies to these three glycoproteins plus many other, as yet unidentified, polypeptides are found in sera derived from patients recovering from VZV infections (8).

We and others have described the fifth ORF (no. 14) with

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the potential to express a glycoprotein (gpV), but a polypeptide(s) expressed from this gene has yet to be described (5, 21). The predicted amino acid sequence bears a limited homology to that of herpes simplex virus (HSV) glycoprotein C (gC), primarily at the carboxy terminus. Unlike gC, the VZV gene possesses unusual in-frame intragenic 42-base-pair repeating sequences that show a strain-dependent copy number variation (21). For example, the Oka strain has four fewer copies of the repeat element than do several wild-type strains (e.g., Scott), with a correspondingly smaller ORF for gpV.

Here we identify the protein products of VZV ORF 14, demonstrate their glycoprotein nature (gpV), and show that antibodies directed against gpV may play a role in virus neutralization. We also present evidence that the LAVV strain Oka is defective in the accumulation of gpV.

MATERIALS AND METHODS

Cells and viruses. The origin and growth of VZV strains have previously been described (21, 30, 33). The vaccinia virus used as parent for the generation of recombinants was strain WR, originally obtained from the American Type Culture Collection. VSC8, a recombinant vaccinia virus containing the β -galactosidase gene under the control of a vaccinia promoter (3), was the kind gift of S. Chakrabarti. All vaccinia viruses were grown and propagated in CV-1 or HFF cells grown in minimum essential medium supplemented with 5% (vol/vol) fetal calf serum (GIBCO Laboratories, Grand Island, N.Y.). Human TK⁻ 143 cells were grown in the same medium containing 25 μ g of 5-bromodeoxyuridine per ml.

Enzymes and chemicals. DNA was digested with restriction endonucleases obtained from Bethesda Research Laboratories, Inc., Gaithersburg, Md.; New England BioLabs, Inc., Beverly, Mass.; or Boehringer Mannheim Biochemicals, Indianapolis, Ind. The Klenow fragment of DNA polymerase I, T4 polynucleotide kinase, T4 DNA ligase, 5-bromo-4-chloro-3-indolyl-D-galactopyranoside, and pre-stained molecular weight marker proteins were obtained from Bethesda Research Laboratories, Inc. 5-bromodeoxyuridine was obtained from Sigma Chemicals Co., St. Louis, Mo. All radiochemicals were purchased from Dupont, NEN Research Products, Boston, Mass. Human antisera derived from patients recovering from VZV infections were the gifts of S. E. Straus, the National Institutes of Health, Bethesda, Md. Human antisera derived from patients who had received LAVV were gifts of A. A. Gershon, Department of Pediatrics, College of Physicians and Surgeons, Columbia University, New York, N.Y.

DNA and recombinant virus isolation. DNA clones containing the *Bam*HI F fragment of VZV strain Scott (21) were digested with restriction enzymes in accordance with the instructions of the manufacturer. Resulting fragments were purified by electrophoresis on and elution from agarose gels by using an electroelution apparatus (model UEA; International Biotechnologies Inc., New Haven, Conn.). A *Bst*NI-*Alu*I restriction fragment containing the complete coding sequence for gpV (gene 14) was then incubated with the Klenow fragment of DNA polymerase I and cloned into the unique *Sma*I site of the vaccinia virus transfer vector pSC11 (Fig. 1) (2). Clones containing gene 14 under the control of the p7.5 vaccinia virus promoter were isolated, and the DNA was purified by CsCl-ethidium bromide gradient centrifugation as previously described (25). CV-1 cells infected with wild-type vaccinia virus were then transfected with calcium

FIG. 1. Construction of plasmids used to derive vaccinia virus recombinants expressing gpV. A representation of the gpV gene and relevant sequences is shown at the top of the figure. Sequences that contain the *Bst*NI and *Alu*I sites used to excise the ORF are shown overlined, and those containing the initiating ATG, the stop codon, and a probable VZV polyadenylation signal are shown underlined. The shuttle vector pSC11 contains the gene for β -galactosidase (*LacZ*, hatched region) under the control of a vaccinia virus promoter (p11) flanked by parts of the vaccinia virus thymidine kinase (*TKL* and *TKR*, filled region) gene. The recombinant plasmid pKIP5 contains the VZV gpV gene (open region) under the control of vaccinia virus promoter p7.5.

phosphate-precipitated DNA from gene 14-containing clones (23, 24). TK⁻ virus was selected by growth on TK⁻ 143 cells in the presence of 5-bromodeoxyuridine, and the resulting recombinants were assayed for blue plaque production in the presence of 5-bromo-4-chloro-3-indolyl-D-galactopyranoside (3). At least three rounds of plaque purification were used for each isolate. Verification that recombinant viruses contained the entire gene 14 insert was obtained by probing gel-fractionated, *Bam*HI-*Eco*RI-restricted whole infected cell DNA immobilized on nitrocellulose (32) with the insert fragment labeled by using nick repair with [³²P]dCTP (3,000 Ci/nmol).

Recombinant vaccinia virus expressing VZV gpI was prepared in a similar fashion, except that a *Bgl*II-*Sma*I fragment containing the entire gpI ORF was isolated, treated with T4 DNA polymerase and nucleotides, and inserted into the *Sma*I site of pSC11. A recombinant virus containing these sequences was isolated (vKIP9) and found to express several heterogeneous polypeptides that reacted with human antisera containing high levels of VZV antibodies. A similar vaccinia virus recombinant expressing products from the gpI gene has previously been described (2).

Analysis of polypeptides. Polypeptides synthesized by HFF cells infected with VZV, wild-type vaccinia virus, or recombinant vaccinia viruses were analyzed by Western immunoblot procedures. Cells were infected with VZV as described previously (21) or with vaccinia viruses at a multiplicity of infection of 2 to 10 PFU per cell. At appropriate times after infection, cells were washed in phosphate-buffered saline and lysed in gel buffer and the polypeptides were electrophoretically separated on 9% (wt/vol) sodium

dodecyl sulfate(SDS)-polyacrylamide gels before transfer to nitrocellulose (29). The blots were then treated with 10% (wt/vol) Carnation instant nonfat dried milk in buffer containing 0.15 M NaCl, 50 mM Tris hydrochloride, pH 7.6, 0.05% (vol/vol) Tween 20, 10 mM EDTA and subsequently reacted with antisera diluted 1:100 in the same buffer containing 0.1% (wt/vol) bovine serum albumin and 1% (wt/vol) dried milk. Bound immunoglobulins were detected with 125 I-labeled *Staphylococcus* protein A (10^5 dpm/ml). Absorbed sera were prepared by mixing infected cell sonic extracts and sera at the ratio of 10^8 cell equivalents per 0.1 ml of antiserum in a manner similar to that described previously (31). Antigen-antibody complexes were removed by high-speed centrifugation prior to use.

Tunicamycin and endoglycosidase treatment. For tunicamycin treatment, vaccinia virus-infected cells were incubated from 4 h after infection until harvest (24 h) in medium containing 1.5 μ g of tunicamycin (Calbiochem-Behring, La Jolla, Calif.) per ml. For endoglycosidase F treatment, cells infected with a vaccinia virus recombinant were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) and the region of the gel containing gpV was excised. Protein was eluted in 10 mM Tris hydrochloride, pH 7.5–1 mM EDTA and incubated with 0.5 U of endoglycosidase F (Boehringer Mannheim) in 0.5% (vol/vol) Triton X-100–0.5% (vol/vol) β -mercaptoethanol–5 mM EDTA and either 100 mM sodium acetate, pH 4.8 (low-pH incubation), or 100 mM potassium phosphate, pH 8.0 (high-pH incubation). After incubation, protein was precipitated with 10% (wt/vol) trichloroacetic acid and Western blotted as described above, following SDS-PAGE.

Production of antisera. Recombinant vaccinia virus was grown and purified as described previously (23, 24). At several sites, 5×10^7 PFU was injected subcutaneously into rabbits, and injection was repeated after 4 weeks; serum was collected at 2-week intervals following the second inoculation and tested for reactivity by Western blot analysis against VZV-infected cells.

Virus neutralization. HFF cells were infected with VZV-infected cells at a 20:1 ratio. After approximately 10% cytopathic effect had developed, the cells were trypsinized and reseeded in the same flask. This step was repeated 24 h later, with the addition of an equal number of uninfected cells. Cells were harvested 24 to 48 h later and ultrasonically disrupted in minimal essential medium with 20% (vol/vol) fetal calf serum. A 0.4-ml portion of sonic extract containing 10^2 to 10^4 PFU was mixed with 0.1 ml of the appropriate antiserum diluted in phosphate-buffered saline. After incubation for 2 h, the mixture was applied to subconfluent HFF monolayers, and plaques were counted after 4 to 6 days.

Indirect immunofluorescence. Subconfluent monolayers of HFF cells were grown on glass cover slips and infected with VZV Scott as described above. Two to four days later, cells were washed three times in ice-cold phosphate-buffered saline and fixed in acetone at -20°C for 10 min. Cover slips were then rehydrated in phosphate-buffered saline containing 10% (vol/vol) fetal calf serum for 15 min at room temperature. Following this, they were incubated with an appropriate dilution of antibody for 1 h and then washed three times with phosphate-buffered saline. Cells were then exposed to goat anti-rabbit immunoglobulin G conjugated with rhodamine (Boehringer Mannheim) for 1 h and washed three times with phosphate-buffered saline. The cover slips were then mounted in 50% glycerol and viewed with a phase-fluorescence microscope (Carl Zeiss Inc., Thornwood, N.J.).

RESULTS

To identify the products of ORF 14, we have used anti-VZV antibodies present in human sera to detect products (gpV) from coding sequences expressed in vaccinia virus recombinants. We have also used antisera to this recombinant as a specific reagent to examine the properties of gpV in VZV-infected cells.

Construction of vaccinia virus recombinants expressing gpV. Figure 1 shows the construction of the plasmid containing the sequences used to create gpV-expressing vaccinia virus recombinants. A plasmid containing the *Bam*HI F fragment from VZV strain Scott DNA was cleaved with *Bst*NI (which cleaves 3 base pairs upstream of the second in-frame ATG of the ORF) and *Alu*I (which cleaves 136 base pairs downstream from the end of the ORF and downstream from a possible polyadenylation signal; Fig. 1). The first ATG in the ORF lies 93 base pairs upstream from the second and has the context 5'TTCCCAATGTCGAAA 3' (5, 21). We considered it more likely that the second ATG, rather than the first, is used because it is immediately followed by a hydrophobic sequence which is the likely signal sequence for gpV; this ATG also has the better Kozak (22) context. This fragment was then inserted into the unique *Sma*I site of pSC11 (3), under the control of the vaccinia virus early-late promoter p7.5. The construction was verified by sequencing the promoter-gpV fusion point (data not shown). The resulting plasmid (pKIP5) also contains *Escherichia coli* β -galactosidase (LacZ) under the control of the vaccinia virus promoter p11. pKIP5 was then transfected into vaccinia virus-infected cells, and several TK⁻ vaccinia recombinants expressing β -galactosidase were isolated. Two of these recombinants were plaque purified and then analyzed for expression from the gpV gene. Both recombinants yielded identical results in the assays to be described.

Expression from gpV-vaccinia virus recombinants. Western blots of infected cells were prepared and reacted with human antisera obtained from patients recovering from VZV infections. Figure 2 shows the reactivity of convalescent-phase antiserum from a zoster patient, with blots derived from equivalent quantities of HFF cells infected with VZV Scott (lane A), uninfected cells (lane B), the vKIP5 vaccinia virus recombinant (lane C), and vSC8, a vaccinia virus recombinant containing pSC8 sequences but no VZV DNA (lane D). The antiserum clearly reacts specifically with polypeptides present in the VZV-infected, but not uninfected, cell extracts. The vaccinia virus recombinant containing the gpV gene expressed polypeptides with molecular weights of 95,000 to 105,000 (95K to 105K polypeptides) which reacted specifically with this and several other convalescent-phase sera, indicating that the proteins expressed from VZV gene 14 are significant immunogens in natural VZV infection. These reactive polypeptides must be products of ORF 14, since the control virus, vSC8, expressed no polypeptides reactive with the VZV convalescent sera.

Rabbits were inoculated with vKIP5 to generate antiserum against gpV, and this serum was used to probe the cellular location of gpV by indirect immunofluorescence. The results are shown in Fig. 3, and they show two samples of VZV Scott-infected cells probed with anti-vKIP5 antiserum (Fig. 3A and B); in both samples, the fluorescence appears to be associated with the plasma membrane and to be patchy in many cells. Both preimmune sera (Fig. 3C) and uninfected cells (Fig. 3D) give faint overall background fluorescence.

The glycoprotein nature of gpV. The above results indicated that the products of gene 14 in VZV Scott are a set of

FIG. 2. Analysis of polypeptides from a vaccinia virus-gpV recombinant. Extracts of cells infected with VZV strain Scott (lane A), uninfected cells (lane B), and cells infected with vaccinia virus recombinants vKIP5 (lane C) and vSC8 (lane D) were electrophoresed, transferred to nitrocellulose, and reacted with a 1/100 dilution of convalescent-phase serum from a zoster patient. Bound antibodies were detected by using ^{125}I -labeled *Staphylococcus* protein A. This figure shows the autoradiogram and gives approximate molecular masses in kilodaltons of a mixture of marker proteins, to the left of lane A.

95K to 105K polypeptides. These are larger than would be predicted from the ORF (61K), but since there are five potential sites for N-linked glycosylation predicted from the sequence (21), this discrepancy is likely to be due to addition of carbohydrate. Two approaches were used to investigate this: *in vivo* tunicamycin treatment and *in vitro* endoglycosidase degradation. These indirect methods had to be used because none of our anti-gpV antibodies works well in immunoprecipitation experiments. In the first approach, HFF cells infected with the vKIP5 were incubated with tunicamycin from 8 to 24 h postinfection and then harvested for Western blot analysis with convalescent-phase serum from a zoster patient. The results (Fig. 4) show that tunicamycin, which inhibits N-linked glycosylation, significantly reduces the formation of 95K to 105K polypeptides, revealing two novel major polypeptides of 64K and 58K and two minor polypeptides of 60K and 68K. We interpret this result to suggest that the 58K polypeptide represents unmodified gene 14 product, while the other species are partially modified; some limited proteolytic digestion may also have taken place. The small amount of mature gpV seen was probably synthesized prior to addition of tunicamycin, since VZV infections have to be carried out with whole cells. Similar blots probed with anti- β -galactosidase antiserum (vKIP5 has an active β -galactosidase gene) showed no difference in either the amount or the size of β -galactosidase as a result of tunicamycin treatment (data not shown).

In the second series of experiments, gpV was isolated from vKIP5-infected cells by elution from polyacrylamide gels and incubated *in vitro* with endoglycosidase F, which trims both N-linked high-mannose simple and low-mannose complex oligosaccharides. Incubation products were concentrated by trichloroacetic acid precipitation, blotted to nitrocellulose after SDS-PAGE, and probed by using a convalescent-phase human serum. The results (Fig. 5) show

FIG. 3. Indirect immunofluorescence in VZV-infected cells using gpV-specific antiserum. HFF cells infected with VZV strain Scott at 2 days postinfection were treated with rabbit anti-vKIP5 antiserum followed by rhodamine-conjugated goat anti-rabbit serum. Shown are two samples of VZV-infected cells treated with anti-vKIP serum (A and B), infected cells with preimmune rabbit serum (C), and uninfected cells with vKIP5 serum (D).

FIG. 4. Effect of tunicamycin on the synthesis of gpV. Cells infected with vKIP5 (vK5) or uninfected (M) were grown in the presence (T+) or absence (T-) of 1.5 μ g of tunicamycin per ml. Proteins were detected by Western blotting after SDS-PAGE by using human anti-VZV serum. The approximate sizes of polypeptides are given to the left of the figure in kilodaltons.

that gpV is trimmed from 95K to 105K to 85K to 90K by endoglycosidase F, under both high-pH (endoglycosidase activity plus peptido-*N*-glycosidase activity)- and low-pH (endoglycosidase activity)-buffered conditions. Similar results were obtained from VZV-infected cell polypeptides after probing with rabbit anti-vKIP5 sera.

Taken together with the tunicamycin results, these data show that the products of VZV gene 14 are glycosylated and contain at least N-linked mannose-type unsialated oligosaccharides.

Comparison of gpV expression from VZV Scott- and Oka-infected cells. Figure 6a shows a Western blot derived from uninfected cells or cells infected with VZV strains Oka or Scott. The blot was probed with human VZV convalescent-phase serum. Approximately 15 to 20 polypeptide species ranging in molecular weight from 40K to $\geq$ 200K are found specifically in both VZV Scott- and VZV Oka-infected cells. Interestingly, however, the patterns for Oka and Scott are not identical (see arrows in Fig. 6a); a consistent difference lies in the 95K to 105K region (see also Fig. 9), and this is the size of gpV. Thus, either Oka makes a smaller gpV than Scott (as we would predict from the smaller size of the Oka ORF), which is masked on the blot, and/or Oka makes only small amounts of gpV. To address this question, we absorbed out antibodies from the human convalescent-phase serum with a large excess of either Scott-infected or Oka-infected cell antigen and tested the residual antibodies in Western blots. Figure 6b shows the reactivity of residual antibodies after absorption with Scott-infected cells; no reactive species are detected. In contrast, absorption with Oka-infected cells leaves antibodies reactive with one major

FIG. 5. Effect of endoglycosidase treatment on gpV. GpV isolated from cells infected with vKIP5 was treated with endoglycosidase F (lanes A and C) in high (lanes A and B)- or low (lanes C and D)-pH buffers. Proteins were detected by Western blotting with human anti-VZV serum, after SDS-PAGE. Numbers to the left of the figure are the approximate sizes of polypeptides in kilodaltons.

species specific to Scott (Fig. 6c, arrow), which is the same size as gpV; other more minor species were inconsistently seen. To verify that this absorbed-out antibody is directed at gpV, it was used to probe Western blots of cells infected with the vaccinia virus ORF 14 recombinant, vKIP5. The results (Fig. 7) show that both the vKIP5 recombinant (lane b)- and Scott (lane d)-infected cells give analogous reactivity at 95K to 105K, strongly suggesting that Oka-infected cell extracts contain very little gpV. As further confirmation of this deficiency in Oka, antiserum was prepared from rabbits infected with the vKIP5 and used as a specific probe for gpV.

FIG. 6. Western blots of infected cells in which human convalescent-phase antisera (a) or antisera absorbed with antigen from cells infected with VZV Scott (b) or VZV Oka (c) were used. Each contains extracts from uninfected cells (M) or cells infected with VZV Scott (S) or Oka (O). Approximate molecular masses for marker proteins are shown to the left of the figure.

FIG. 7. Western blots of cell extracts. Polypeptides from cells infected with wild-type vaccinia virus (a), the vaccinia-gpV virus recombinant vKIP5 (b), uninfected cells (c), VZV Scott (d), or VZV Oka (e) were separated on SDS-PAGE. Western blots were reacted with 1:100 dilutions of human sera absorbed with excess antigen from cells infected with VZV Oka as described in the text. The approximate molecular masses of a mixture of marker polypeptides are shown in kilodaltons on the right.

Identical Western blots of Scott, Oka, and uninfected cell polypeptides were reacted with either a VZV convalescent-phase human serum (Fig. 8a) or with the anti-vKIP5 serum (Fig. 8b). As expected, anti-vKIP5 antibodies bound specifically to 95K to 105K polypeptides present in Scott-infected cells, but not in uninfected cells. In VZV Oka-infected cells, however, only trace amounts of gpV of the smaller size expected from the ORF are visible. These data, therefore, all point to defective accumulation of gpV in VZV Oka-infected cells.

VZV neutralizing activity in gpV-specific antisera. We have been able to demonstrate, by using purified virion preparations, that gpV is present in the VZV particle (J. Hougland and P. R. Kinchington, unpublished observations). This raises the possibility, which could be tested with the recombinant-derived rabbit antiserum, that gpV might serve as a VZV-neutralizing antigen. The antiserum was mixed with VZV preparations and plated on HFF cells for infectivity assays. After 4 days, cells were fixed and plaques were counted; for comparison, both rabbit preimmune sera and convalescent-phase serum from a zoster patient were included in the assay. As can be seen in Table 1, preimmune serum had a slight effect on VZV infectivity, at the highest dilution. In contrast, both human serum and gpV-specific rabbit serum substantially affected the infectivity of VZV Scott, reducing the titers by greater than 90% at dilutions up to 1/25 and by more than 50% at a dilution of 1/125. Inclusion of guinea pig complement with anti-gpV antiserum in the assay had no effect on neutralization of VZV. As predicted, VZV Oka was neutralized substantially less well than Scott by the anti-gpV serum, whereas human serum neutralizes both viruses to similar extents. These results imply that gpV

FIG. 8. Western blots of VZV-infected cell proteins. Uninfected HFF cell (U) and Scott (S)- and Oka (O)-infected cell proteins were separated by PAGE and blotted to nitrocellulose. In panel a, blots were reacted with a VZV convalescent-phase human serum, while in panel b, blots were reacted with rabbit anti-gpV antiserum. In both cases, reaction was detected with 125 I-labeled *Staphylococcus* protein A. The figure shows the autoradiograms, with molecular masses of protein markers given in kilodaltons to the right of panel b.

is a target for complement-independent virus-neutralizing antibodies and that the level of neutralization of VZV Oka reflects small amounts of gpV in VZV Oka virions.

Reactivity of sera from vaccinees to gpV. The above data show that VZV Oka accumulates only trace levels of gpV compared with a wild-type strain. Recently we have shown that wild-type VZV strains in general, including several Japanese isolates, accumulate levels of gpV similar to that of Scott (P. R. Kinchington, P. Ling, M. Pensiero, A. Gershon, J. Hay, and W. T. Ruyechan, in C. Lopez, M. Mori, B. Roizman, and R. Whiteley, ed., *Proceedings of the 4th International Meeting on the Immunobiology and Prophylaxis of Human Herpesviruses*, in press). This gave us the opportunity to investigate the use of gpV as a specific marker for the LAVV (Oka) in clinical studies; no equivalent reagent is currently available.

TABLE 1. VZV neutralizing activity in gpV-specific antisera^a

Antiserum	% Survival with the following serum dilutions ^b :		
	1:5	1:25	1:125
Preimmune serum	70	85	100 (Scott)
	70	86	100 (Oka)
Human serum	0 ^c	8	36 (Scott)
	0	0	16 (Oka)
Anti-gpV serum	0	13	36 (Scott)
	19	76	100 (Oka)

^a Dilutions of antisera were mixed with preparations of VZV-infected cells containing about 100 PFU of virus. After 2 h at room temperature, the mixtures were plated on HFF cells, and they were fixed after 6 days.

^b Numbers are the percent survival of plaques relative to a control plate untreated with serum.

^c 0, No plaques visible.

FIG. 9. Western blots of extracts derived from uninfected cells or cells infected with VZV Scott or VZV Oka (a and b, lanes 3, 2, and 1; c and d, lanes 1, 2, and 3) or vaccinia virus viruses expressing VZV gpI, no VZV genes, or VZV gpV (lanes 4, 5, and 6). Each set of six lanes was probed with a VZV human convalescent-phase serum (MH) (a) or with sera derived from Oka vaccinees (b, c, and d) as described in the text. Approximate molecular masses of marker proteins are indicated in kilodaltons.

As a control, a number of sera from LAVV vaccinees were tested for reactivity to whole VZV-infected cell polypeptides on Western blots; three examples are shown in Fig. 9. Sera gave variable responses in this test, and the presence of antibodies to gpV (or to other specific proteins) could not be assessed with any confidence, although some antisera gave strong responses (see, for example, Fig. 9b, lanes 1 and 2). We have consistently observed that sera from natural infections appears more reactive than that from vaccinees, particularly in the <45K polypeptide range (compare Fig. 9a, lanes 1 and 2, with 9b, lanes 1 and 2, for example). However, when the sera were tested against recombinant gpV proteins, the results were well defined and interesting. With the wild-type antiserum, there is a strong response against gpV (Fig. 9a). Of the three vaccinees' sera shown, two antisera showed very low levels of antibodies to gpV (Fig. 9c and d) but a third appeared positive for gpV (Fig. 9b). Examination of the clinical history of those patients with LAVV sera positive for gpV suggested a correlation with vaccine-related problems; specifically, the serum in Fig. 9b was derived from a patient who had shown vaccine-induced zoster (A. A. Gershon, personal communication). As an internal control in all of these assays, we constructed and used a VZV gpI-expressing vaccinia virus recombinant (Oka is not defective in gpI accumulation); all of the sera tested showed anti-gpI activity (Fig. 9, lanes 4).

DISCUSSION

Five glycoprotein genes have been identified in the VZV genome, and the products of four of these genes have already been described in varying detail. In this report, we have identified products of the fifth glycoprotein gene, ORF 14 (gpV), as a set of glycosylated polypeptides of about 95K to 105K, which are present in VZV particles. The sizes of proteins made from the gpV gene in vaccinia virus recombinant-infected cells and in VZV-infected cells are similar (95K to 105K) but, with vaccinia virus, the bias is towards the 95K range, while authentic VZV gpV is mainly around 105K (Fig. 7). This may reflect less or somewhat different glycosylation in vaccinia virus-infected cells.

Several useful reagents have been generated in the course of this project, among which are vaccinia virus recombinants expressing specific VZV proteins and antisera directed against them. By using these reagents, we have examined several characteristics of gpV, including glycosylation. The

effect of tunicamycin and the susceptibility to digestion with endoglycosidase F confirm that at least part of the glycosylation is N linked, although we are unable to say whether all five potential glycosylation sites are used. Glycosylation of gpV may also include O-linked carbohydrates, and this is inferred from the data on tunicamycin and endoglycosidase F treatments. For example, tunicamycin treatment results in the appearance of two novel bands close to the predicted size for ORF 14; such polypeptides, since they are not cotranslationally modified, are unlikely to be transported to the Golgi where O-linked polysaccharide addition is likely to occur. In contrast, the digestion with endoglycosidase F of N-linked sugars from mature species results in novel polypeptides significantly larger than the predicted size of unglycosylated protein; thus, some glycosylation may be resistant to endoglycosidase F, and one possibility is that this is O linked. Many herpesvirus glycoproteins are O glycosylated, including HSV-1 gC, to which gpV is partially related. We do not yet know whether gpV contains further modifications, such as phosphorylation, as has been demonstrated for VZV gpI (14, 17).

Many herpesvirus glycoproteins are targets for neutralization by circulating antibodies. We show here that anti-gpV antibodies are able to neutralize VZV in the absence of complement, suggesting that gpV is exposed on the virion envelope; in addition, we demonstrate that gpV is a natural immunogen, since antibodies to gpV are present in sera from VZV convalescent patients. In this sense, gpV is similar to its other herpesvirus homologs; e.g., the HSV gC and the pseudorabies virus gpIII are major neutralization targets. Another feature which gpV shares with HSV gC is its intracellular location. Indirect immunofluorescence with anti-vKIP5 antisera shows patchy plasma membrane fluorescence in VZV-infected cells reminiscent of that shown by anti-HSV gC sera in HSV-infected cells (9).

A surprising finding in this report is the lack of gpV associated with the VZV vaccine strain, Oka. Several lines of evidence demonstrate this: (i) antibodies in human sera directed against gpV are not blocked by excess antigen from VZV Oka-infected cells under circumstances in which essentially all other reactive antibodies are absorbed out, (ii) sera prepared against gpV expressed by vaccinia virus recombinants react with polypeptides in VZV Scott (and a range of other wild-type VZV strains) but react only weakly with polypeptides in VZV Oka, and (iii) these sera neutralize

VZV Oka much less efficiently than VZV Scott. A fourth line of evidence for a low level of gpV accumulation in LAVV is that sera from many vaccine recipients contain much lower amounts of gpV antibodies than those coping with a wild-type infection, although equivalent levels of antibodies to gpI are found in both populations. Conceivably, an anti-gpV antibody test might be useful as a diagnostic marker for Oka infection; however, a commercially viable test would have to be made much more sensitive than the Western analyses shown here.

The most likely reason for the Oka defect is that it simply makes less (or keeps less) gpV than other strains, and it should be noted that HSV gC (the partial homolog of gpV) is nonessential for virus growth (7, 18). Alternatively, however, the Scott-Oka gpV divergence could be the result of major antigenic differences between gpV in these two viruses, rather than a quantitative difference in accumulation, although we feel that this is unlikely to be the case. The molecular basis for the gpV deficit in Oka strains is currently being investigated. Our preliminary observations suggest that Oka has a specific defect in transcription from the gpV gene (P. Ling, P. R. Kinchington, W. T. Ruyechan, and J. Hay, unpublished data), but we do not yet know the precise molecular basis for the defect; considerable work is currently underway on this aspect of Oka.

During the screen of vaccinees' sera, one was shown to have antibodies to gpV. One explanation for this could be an anamnestic response to gpV, through a previously unreported (or undetected) wild-type infection with VZV. Another more interesting possibility arises from the fact that the patient with anti-gpV antibodies had a typed case of vaccine-associated zoster, although his virus was clearly Oka (typed through DNA restriction analysis; A. A. Gershon, unpublished observations). A follow-up study from our laboratory has demonstrated that the Oka virus isolated from this vaccinee expressed gpV at wild-type levels (P. R. Kinchington, unpublished observations). These data raise the possibility of a relationship between gpV expression and attenuation in which a gpV⁺ phenotype may be more virulent than a gpV⁻ phenotype. In order to address this question satisfactorily, we plan to examine a large number of sera and virus isolates from normal and vaccinated individuals, with particular reference to clinical status and gpV antibody levels.

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