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Identification and Characterization of Murine Gammaherpesvirus 68 gp150: a Virion Membrane Glycoprotein

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Murine gammaherpesvirus 68 (MHV-68) is a naturally occurring virus of murid rodents which displays pathobiological characteristics similar to those of other gammaherpesviruses, including Epstein-Barr virus (EBV). However, unlike EBV and many other gammaherpesviruses, MHV-68 replicates in epithelial cells in vitro and infects laboratory strains of mice and therefore provides a good model for the study of gammaherpesviruses. Studies of sequences around the center of the MHV-68 genome identified a gene (designated BPRF1 for *Bam*HI P fragment rightward open reading frame 1) whose putative product had motifs reminiscent of a transmembrane glycoprotein. All other gammaherpesviruses have a glycoprotein in this genomic position, but the BPRF1 gene showed sequence homology with only the EBV membrane antigen gp340/220. Biochemical analysis showed that the product of BPRF1 was a glycoprotein present on the surface of infected cells, and immunoelectron microscopy showed that it was present in the virus particle. In addition, antibodies to the BPRF1 product raised by using a bacterial fusion protein neutralized the virus in the absence of complement. The predominant molecular weights of the protein were 150,000 and 130,000. Pulse-chase analysis and endoglycosidase-H digestion showed that the 130,000-molecular-weight form was a precursor of the 150,000-molecular-weight form, and cell surface labelling showed that the 150,000-molecular-weight form alone was on the cell surface. We therefore named the protein gp150. Since gp150 is the first virion-associated glycoprotein and neutralizing determinant of MHV-68 to be characterized, it provides a valuable tool for the future study of virus-host interactions.

Murine gammaherpesvirus 68 (MHV-68) is a pathogen of murid rodents and was first isolated from *Clethrionomys glareolus* (bank vole) in Slovakia (3, 4, 38). It was classified as a gammaherpesvirus on the basis of both genetic and biological analyses. Thus, sequence analysis of limited regions of the genome revealed homologous open reading frame (ORFs) which have the same genetic organization as other gammaherpesviruses (9, 10, 32). After intranasal infection of mice, MHV-68 replicates in epithelial cells in the lungs and spreads to lymphoid tissue, where it infects B lymphocytes (35, 36). A splenomegaly which is self-limiting and results in long-term viral persistence in B cells ensues (35, 36). The control of initial infection appears to involve a critical role for a CD8 T-cell response (11). In addition, MHV-68 forms plaques readily in established cell lines and infects and persists in B-cell lines in vitro (37). This virus is therefore attractive for use in a small-animal model for the study of the interaction of gammaherpesviruses with hosts.

Herpesviruses express a number of transmembrane glycoproteins which are involved in virion structure as well as binding to and entry of the virus into cells (28). These proteins are generally N and/or O glycosylated and are expressed on the cell surface as well as on the virion. Many functions of these glycoproteins in the alphaherpesviruses have been elucidated (28), and some of these proteins, notably glycoprotein B (gB), gH, and gL, have known homologs in the gammaherpesviruses (13, 14, 24, 45). However, most of the glycoproteins encoded by the gammaherpesviruses appear to be unique to this subfamily (16, 18, 19, 22, 26, 34, 40).

An important function of virion glycoproteins involves binding of the virus to a cellular ligand. Glycoproteins with this function have been identified in two gammaherpesviruses, bovine herpesvirus type 4 (BHV-4) and Epstein-Barr virus (EBV). BHV-4 gp8 mediates adsorption to cells via heparin on the cell surface (8, 42), whereas in EBV, gp340/220 binds to CR2 (CD21), a predominantly B-lymphocyte-expressed protein which mediates EBV adsorption and endocytosis (23). As a consequence, antibodies to both gp8 and gp340/220 are virus neutralizing in the absence of complement (8, 16). The gene for BHV-4 gp8 has not been mapped, but the gene for EBV gp340/220 is located near the center of the virus genome in the *Bam*HI L fragment (2). It is also known that the smaller gp220 form is the product of an alternatively spliced message (41).

Little is known about the glycoproteins of MHV-68, and the only one to have been characterized to date is the homolog of gB (32). During the sequencing of the center of the MHV-68 genome, a gene with consensus glycoprotein motifs was identified (25). This gene was in the same relative position and orientation in the MHV-68 genome as the gene encoding gp340/220 is in EBV. Since EBV gp340/220 is involved in binding to host cells, we considered that analysis of the product of this gene might give valuable information on the mechanism of MHV-68 infection. In this paper, we show that the MHV-68 gene is expressed as a transmembrane glycoprotein which is a component of the virus particle and is a neutralizing determinant.

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MATERIALS AND METHODS

Virus and cells. Working stocks of MHV-68 clone g2.4 were prepared in BHK-21 cells as described by Efstathiou et al. (10). C127 (ATCC CRL 1616) is a mouse epithelial cell line which was derived from a mammary carcinoma (21) and was grown in Dulbecco's modified Eagle medium (Gibco/BRL, Paisley, United Kingdom) containing 10% fetal calf serum (Globepharm, Esher, United Kingdom).

DNA cloning and sequencing. All molecular cloning techniques were performed as described by Sambrook et al. (27). The MHV-68 *Eco*RI A fragment to the rightward *Hin*dIII site was obtained from the pUC13 library described previously (10). Small-scale plasmid DNA preparations were made with Wizard Minipreps (Promega), and double-stranded templates were sequenced by using fluorescent dideoxy chain termination chemistry (PRISM kit; Perkin-Elmer) and analyzed with an ABI373A sequence machine (Perkin-Elmer). Overlapping sequence of the complete ORF was obtained for both strands by the use of oligonucleotide primers. The sequence was assembled and analyzed with the Genetics Computer Group (GCG) suite of computer programs (7).

A cDNA corresponding to BPRF1 was cloned by reverse transcription-PCR (RT-PCR) as follows. Cytoplasmic RNA was extracted from C127 cells 18 h after infection with MHV-68. Total RNA (1 μg) was then reverse transcribed with Moloney murine leukemia virus reverse transcriptase (Promega) and primer 5′-GAG CTC GAA TTC CAG CTG $(T)_{15}$-3′ (antisense) as described previously (27). This oligonucleotide was designed to prime cDNA synthesis from the poly(A) tract and generate an *Eco*RI site after PCR amplification. cDNA was then amplified by PCR using the same antisense primer and a second oligonucleotide, 5′-CGC GAA GCT TAT GTG TGG CGT TAA ATC CCT AGC -3′ (sense). This primer contains sequences around the putative translational start of the gene with a *Hin*dIII site at the 5′ end. PCR amplification with *Pfu* DNA polymerase (Stratagene) was performed for 35 cycles of 94°C for 1 min, 35°C for 1 min, and 72°C for 4 min. Amplified product was then cut with enzymes *Hin*dIII and *Eco*RI and cloned into the vector pSP72 (Promega). Sequencing of the cDNA revealed one nucleotide difference from the genomic sequence (25); however, this change was silent and therefore deemed not important.

Antisera. Monospecific anti-MHV gp150 was generated as follows. A portion of BPRF1 corresponding to nucleotides 904 to 1387 (as shown below [see Fig. 1]) was amplified by PCR as described above using the primers 5′- GTA GGA TCC GTG AGA GTG TAC ACA AAG ACG C-3′ (sense) and 5′-GGA GAA TTC TCC TTT GGT TCA GC-3′ (antisense). The product was cut with the enzymes *Bam*HI and *Eco*RI (sites incorporated into the sense and antisense oligonucleotides, respectively) and was cloned into the bacterial expression vector pGEX-3X (Pharmacia) between these two restriction sites. A glutathione *S*-transferase–gp150 fusion protein with an approximate molecular weight of 66,000 was expressed in *Escherichia coli* and was purified on glutathione-Sepharose beads according to the manufacturer's instructions. Purified fusion protein was then used to immunize rabbits in combination with Hunter's Titermax adjuvant (CytRX, Norcross, Ga.). Serum was prepared from blood samples taken before and after immunization.

Rabbit anti-MHV-68 antiserum was prepared by using MHV-68-infected rabbit kidney cells as described previously (35).

Radioimmunoprecipitation. Cells were metabolically labelled with [^{35}S]methionine or [^{14}C]glucosamine and proteins were immunoprecipitated and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with 5.5% polyacrylamide gels exactly as described previously (33). Pulse-chase analysis with a labelling time of 15 min in methionine-free medium was performed. The cells were then washed with medium containing nonradioactive methionine, and the cells were incubated for the times indicated in Fig. 3 before being harvested. For MHV-68, cells were infected at 10 PFU per cell and infection was allowed to proceed for 18 h prior to labelling. For vaccinia virus, the cells were infected at 30 PFU per cell and labelled 3 h postinfection. Antisera were used at a 1/200 dilution. Endoglycosidase H (endo-H) digestion was performed with 3 mU of recombinant enzyme (Boehringer) as described previously (32).

In vitro transcription and translation. Coupled transcription and translation reactions were performed by using a rabbit reticulocyte lysate system and SP6 polymerase (Promega). Reactions were carried out according to the manufacturer's instructions, incorporating [^{35}S]methionine and using 1 μg of substrate plasmid DNA. Products were analyzed by SDS–5.5% PAGE and autoradiography exactly as described previously (33).

Cell surface biotinylation. Cell surfaces were biotinylated by using D-biotin-*N*-hydroxysuccinimidester (Boehringer) as described by Crepaldi et al. (6). The cells were then lysed and immunoprecipitated with antibodies, and proteins were separated by SDS-PAGE and Western blotted (immunoblotted) as described previously (32). Biotinylated proteins were then detected by using alkaline phosphatase-conjugated streptavidin (Boehringer) and an enhanced chemiluminescence kit (Amersham) according to the manufacturer's instructions.

Immunofluorescence analysis. Cells were grown and infected on microscope slides and either fixed in acetone for 3 min at −70°C prior to being stained or stained live without fixation but with the addition of azide throughout the staining procedure. The staining procedures were done as described previously (30, 31). Primary antibodies were used at a dilution of 1/250. Reactivity was detected with fluorescein isothiocyanate-conjugated swine anti-rabbit immunoglobulins (Dakopatts) at a dilution of 1/30.

Immunoelectron microscopy. C127 cells were pelleted and fixed in 4% paraformaldehyde–0.1% glutaraldehyde in phosphate-buffered saline (PBS) (pH 7.4) at 20°C. After briefly being rinsed in buffer and then distilled water, specimens were dehydrated sequentially in ethanol at low temperature as follows: 30% for 30 min at 0°C and then 50, 70, 95, and 100% for 1 h each at −20°C. The specimens were then embedded in Lowicryl HM20 resin (Agar Scientific, Stanstead, Essex, United Kingdom) at −30°C for 14 h in conical capsules. Embeddings were polymerized with diffuse UV (two TL6W/08 tubes [Philips], 365-nm wavelength, indirect illumination at 20- to 30-cm distance) for 48 h at −30°C.

Sections (60 nm thick) were mounted on 300-mesh gold grids, rinsed for 1 h in 10% normal goat serum in PBS, and then incubated for 2 h in either preimmune rabbit serum or rabbit anti-gp150 diluted 1/100 in PBS. After being rinsed with buffer, specimens were incubated with goat anti-rabbit immunoglobulin G conjugated to 5-nm-diameter colloidal gold (Amersham International) diluted 1/25 in Tris-buffered saline (20 mM Tris-HCl, 150 mM NaCl; pH 8.2). After further buffer rinsing, specimens were dipped in distilled water and dried. The grids were subsequently stained with uranyl acetate and lead citrate and examined at 60 or 80 kV in an EM400 transmission electron microscope (Philips).

Virus neutralization assay. Sera were heat treated at 56°C for 30 min to destroy complement. Serum dilutions of between 1/10 and 1/100 were added to 100 PFU of MHV-68, and the mixture was incubated for 1 h at 37°C. The virus-serum combination was then used to infect subconfluent C127 cells. After 5 days, the monolayers were fixed and plaques were counted. Each serum concentration was assayed in quadruplicate on three separate occasions with comparable results.

Nucleotide sequence accession number. The nucleotide sequence of BPRF1 has been submitted to the Genome Sequence Database (GSDB) and has been assigned accession no. L47321.

RESULTS

Sequence of the BPRF1 gene. The sequence of a potential transmembrane glycoprotein was identified as part of a larger project to sequence the *Eco*RI A fragment of MHV-68 (37). Since this is the first rightward ORF in the *Bam*HI P fragment, we have named it BPRF1 after the convention observed for EBV (2).

The DNA sequence of BPRF1, the predicted amino acid sequence, and the sites of consensus motifs are shown in Fig. 1. 5′ of the ORF, there were two TATA boxes which could act as promoter elements for the gene. At the 3′ end of the gene, there was a consensus polyadenylation signal sequence. A cDNA corresponding to BPRF1, 1.5 kbp long, was synthesized by RT-PCR as described in Materials and Methods, using an oligo(dT) primer at the 3′ end and a gene-specific primer which overlapped the predicted translational initiation site at the 5′ end. Only one product, 1.5 kbp long, was produced from the RT-PCR. Three independent cDNA clones were obtained from this product, all of which had the same restriction fragment patterns, which were identical to those predicted from the genomic sequence, and the sequence of the cDNA corresponded exactly to the genomic sequence. The length of this cDNA corresponds well with the single 1.6-kb late viral RNA transcribed across this ORF (29), suggesting that the cDNA represents the complete transcription unit and that there is no splicing. Of interest also is the fact that the polyadenylation signal used forms part of the end of the ORF.

Computer-assisted analysis of the predicted amino acid sequence using the algorithms of Engelman et al. (12) revealed two potential hydrophobic transmembrane domains. The first of these (residues 1 to 18) displayed motifs consistent with it being a signal peptide. The position of a potential signal peptidase cleavage site, present at the end of the underlined sequence in Fig. 1, was determined by the method of von Heijne (43). Cleavage of the signal peptide would yield a potential mature core protein of 465 residues with a molecular weight of 48,000. The second hydrophobic domain (residues 454 to 473) was of sufficient length to be a transmembrane anchor domain. Situated between these two hydrophobic domains were three potential sites for the addition of N-linked glycosylation; however, two of these motifs contain a proline residue and are

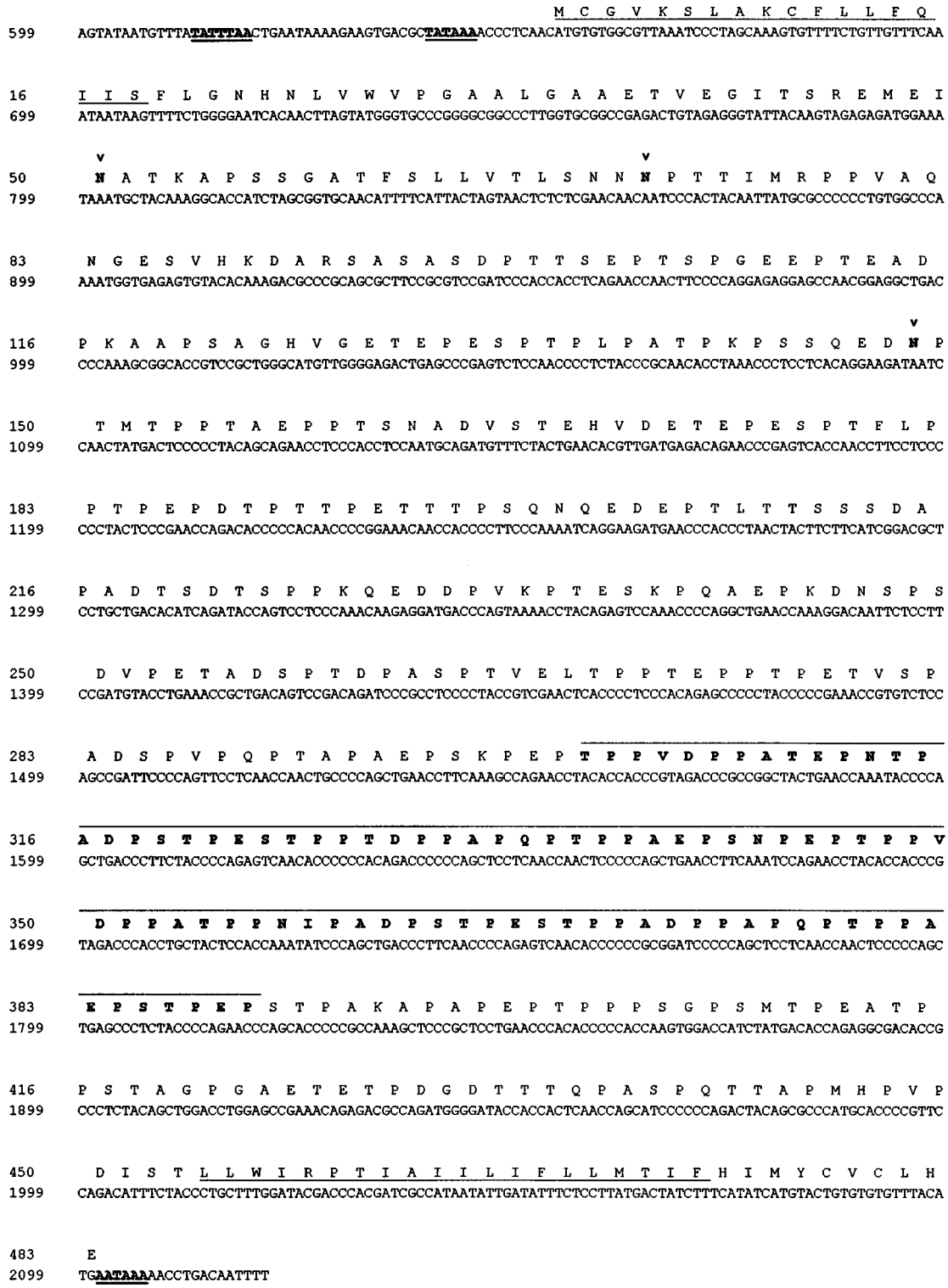

FIG. 1. Sequence of the MHV-68 gp150 ORF BPRF1. The sequence of part of the *Eco*RI fragment which contains BPRF1 is shown. The DNA sequence is numbered from the upstream *Bam*HI site which is the boundary between the *Bam*HI P_2 and N fragments according to the map of Efstathiou et al. (10). The end of the sequence represents the end of the cDNA obtained by RT-PCR. The predicted amino acid sequence is shown above the DNA sequence. The sites of the consensus promoter and polyadenylation signal sequences upstream and downstream of the ORF (boldface and underlined), the extent of the two hydrophobic domains (underlined), the sites of potential N-linked glycosylation (boldface and marked "v" above the residues concerned), and the extent of the proline-rich 22-amino-acid repeat unit (boldface and overlined) are indicated.

TABLE 1. Comparison of murine gammaherpesvirus gp150 with positional homologs from other gammaherpesviruses

Virus	Glycoprotein	No. of residues	Optimized FASTA score[a]
MHV-68	BPRF1/gp150	483	2,087
EBV	gp340	907	278
BHV-4	BORFD1	273	128
Herpesvirus saimiri	Gene 51	269	37
Equine herpesvirus 2	Gene 51	281	37
EBV	BZLF2	223	30

[a] Comparisons of protein sequences were made by using the FASTA option of the University of Wisconsin GCG package relative to the MHV-68 gp150.

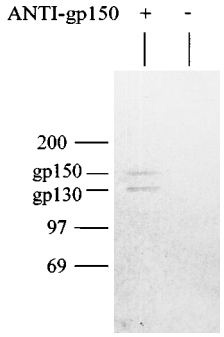

FIG. 2. Glycosylation of gp150. Cells (C127) were infected with MHV-68 at 10 PFU per cell for 16 h and then metabolically labelled with [^{14}C]glucosamine. Proteins were immunoprecipitated with either preimmune serum (−) or anti-gp150 (+). The positions of molecular weight markers (in thousands) are shown on the left.

therefore unlikely to be utilized. Analysis of the amino acid composition of the protein revealed a high content of serine and threonine residues (24%), so there also existed the potential for the protein to be highly O glycosylated. The molecule was also predicted to have an unusually high proline content (23%), with a concentration in a proline-rich repeat domain (residues 302 to 389). This is an imperfect repeat consisting of four copies of the 22-residue motif T P P X D P P A (T or P) X P (N or T) X P A (D or E) P S (T or N) P E (S or P).

In the gammaherpesviruses for which sequence data are available (herpesvirus saimiri, equine herpesvirus 2, BHV-4, and EBV), a potential glycoprotein gene has been found at this locus (1, 2, 20, 39). Indeed, EBV has two glycoprotein genes, BZLF2 and the gp340/220 gene, as well as the EBNA3 family of genes in the same region (2). Comparisons of the predicted amino acid sequence of BPRF1 with positionally analogous glycoprotein sequences from other gammaherpesviruses were performed with the FASTA option of the GCG package (7). The results are shown in Table 1. The only gene product with any significant homology to the product of BPRF1 is EBV gp340. This protein is much larger than the BPRF1 product, and the similarity to the BPRF1 protein occurs in the carboxy-terminal 500 amino acid residues. Further analysis using the GAP option of the GCG package showed that there were 25% identity and 40% similarity at the amino acid level between the BPRF1 product and EBV gp340. Of interest is the fact that like the BPRF1 product, gp340 has a proline-rich repeat domain whose repeat units are 21 residues long. This structural motif, taken together with sequence homology and genetic location, suggests that BPRF1 and gp340 may be derived from a common ancestral glycoprotein.

These data indicate that MHV-68 has a gene which has structural motifs reminiscent of a type I membrane-bound glycoprotein and that this gene may be a distant homolog of the EBV gp340 gene.

The product of BPRF1 is a glycoprotein. To analyze the product of BPRF1, we first raised monospecific antibodies in rabbits, using a portion of the gene product (amino acid residues 86 to 244) fused to glutathione *S*-transferase as an antigen as described in Materials and Methods. The expression of BPRF1 was then analyzed by radioimmunoprecipitation from [^{14}C]glucosamine-labelled cells.

Figure 2 shows that two proteins with apparent molecular weights of 150,000 and 130,000 were precipitated specifically from [^{14}C]glucosamine-labelled, MHV-68-infected cells by anti-BPRF1 antibodies. These results indicate that the product of BPRF1 is a glycoprotein with two forms with molecular weights of 150,000 and 130,000. We have named these proteins gp150 and gp130, respectively.

gp130 is a precursor form of gp150. A number of herpesvirus glycoproteins have precursor and mature forms with two distinct molecular weights (5, 28, 44). We investigated the possibility that gp130 was the precursor of gp150, using a number of approaches. First, we performed pulse-chase analysis. Second, we studied the effect of endo-H on the proteins. Third, we performed in vitro transcription and translation on the cDNA. Finally, we analyzed the molecular weight of the protein present at the surface of infected cells.

For the pulse-chase experiment, cells were infected for 16 h and then metabolically labelled for 15 min with [^{35}S]methionine. Excess label was washed away, nonradioactive methionine was added, and the cells were then sampled at various times. Proteins were immunoprecipitated with anti-gp150 and analyzed by SDS-PAGE. The results (Fig. 3) showed that immediately after the pulse, gp130 predominated. However, over the period of the chase, the relative intensity of the gp130 band decreased, whereas that of the gp150 band increased. These results are consistent with the pool of labelled gp130 being converted into gp150 and suggest that gp130 is the precursor of gp150. An additional, less intense band with a molecular weight of 110,000 (termed p110) was also observed in this experiment. The level of labelled p110 did not appear to decrease similarly to gp130, implying that it was not converted into the higher-molecular-weight forms over this time course. This protein was not glycosylated (Fig. 2) but was observed on

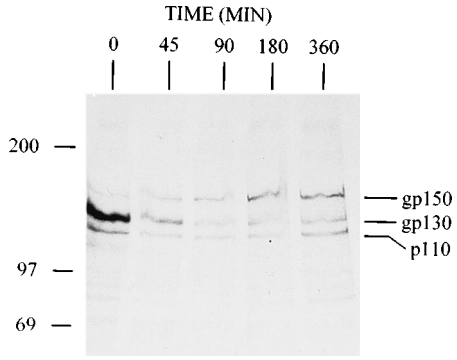

FIG. 3. Pulse-chase analysis of gp150 expression. Cells (C127) were infected with MHV-68 at 10 PFU per cell for 16 h and then metabolically labelled with [^{35}S]methionine for 15 min. The cells were then washed, medium containing cold methionine was added, and the cells were harvested at the indicated times afterwards. Proteins were immunoprecipitated by anti-gp150 and analyzed by SDS-PAGE and autoradiography. The positions of molecular weight markers (in thousands) are shown on the left, and the positions of the predominant bands are marked on the right.

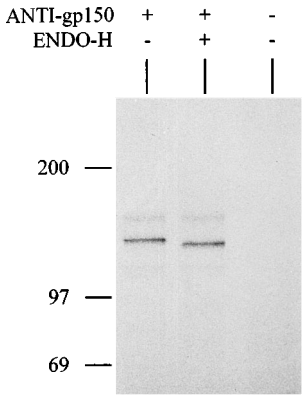

FIG. 4. Endo-H sensitivity of MHV-68 gp130. Cells (C127) were infected with MHV-68 at 10 PFU per cell for 16 h and then metabolically labelled with [^{35}S]methionine. Proteins were immunoprecipitated with either preimmune serum (−) or anti-gp150 (+). An aliquot of the proteins immunoprecipitated by anti-gp150 was exposed to endo-H (+). The positions of molecular weight markers (in thousands) are shown on the left.

Western blots of MHV-68-infected-cell extracts (not shown). Therefore, p110 represents a nonglycosylated cotranslational product of BPRF1.

To confirm that gp130 was a precursor form, immunoprecipitated proteins were treated with endo-H. This enzyme digests only immature N glycans of the high-mannose type, leaving more-processed glycosylation products intact, and can thus be used to diagnose precursor forms of glycoproteins localized in the endoplasmic reticulum (17). Cells were metabolically labelled with [^{35}S]methionine between 16 and 20 h postinfection, radiolabelled proteins were immunoprecipitated with either anti-gp150 or preimmune serum, and a portion of the immunoprecipitate was treated with endo-H. The results (Fig. 4) showed that no labelled proteins were precipitated from MHV-68-infected cells with preimmune serum. Anti-gp150 immunoprecipitated gp130 and gp150 from MHV-68-infected cells. p110 was also immunoprecipitated by anti-gp150, but the band was extremely faint and did not survive photographic reproduction. After digestion with endo-H, gp130 was reduced in size to a molecular weight of around 125,000, whereas gp150 remained unaffected. Thus, gp130 but not gp150 contained immature, high-mannose carbohydrate, suggesting that the former is the precursor of the latter. The endo-H-digested product of gp130 should represent the unglycosylated core of the protein. The small difference in molecular weight is consistent with the small number of sites available for N-linked glycosylation.

The apparent molecular weight of the unglycosylated core was much greater than the predicted molecular weight of the primary translation product. To confirm that no other posttranslational processing was responsible for the anomalous migration of the protein and to study the nature of p110, we performed in vitro transcription and translation on the gp150 cDNA. The results (Fig. 5) showed that no labelled products were observed when the vector alone (pSP72) was used and that two bands with approximate molecular weights of 110,000 and 125,000 were seen when the vector containing the gp150 cDNA (pSP72/gp150) was used. This confirms that the protein backbone of gp150 migrates at an anomalously high apparent molecular weight and that the endo-H-digested product described above represents the unglycosylated core. In addition, this confirms that p110 is a related cotranslational product of this ORF.

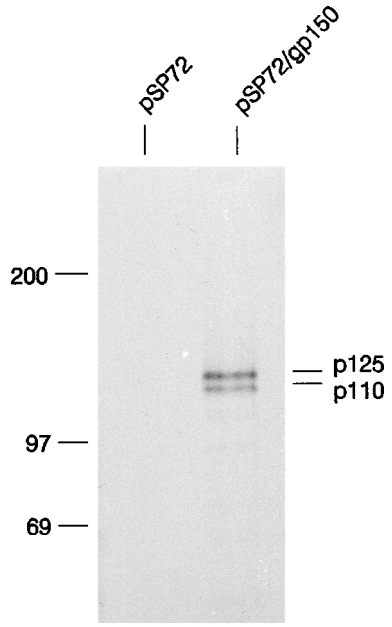

FIG. 5. In vitro transcription and translation of the gp150 cDNA. Coupled transcription translation reactions were performed with a rabbit reticulocyte lysate system and SP6 polymerase on either vector alone (pSP72) or vector containing the gp150 cDNA (pSP72/gp150). Reactions incorporating [^{35}S]methionine were carried out, and products were analyzed by SDS-PAGE followed by autoradiography. The positions of molecular weight markers (in thousands) are shown on the left.

Finally, we analyzed the molecular weight of the protein present on the surface of infected cells. Cells were infected for 16 h and then surface labelled with biotin. Proteins were immunoprecipitated, and then biotinylated proteins were analyzed by Western blotting. The results (Fig. 6) showed that in the absence of virus or specific antibody, no bands were seen.

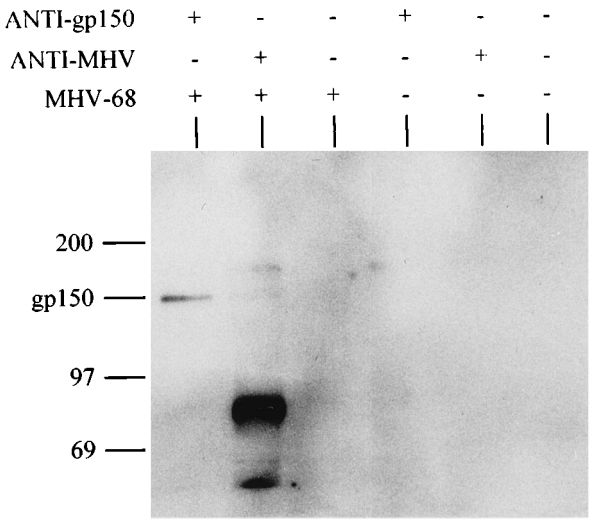

FIG. 6. Cell surface analysis of MHV-68-infected cells. Cells (C127) were either mock infected (−) or infected (+) with MHV-68 at 10 PFU per cell for 16 h and then surface labelled with biotin. Proteins were then precipitated with preimmune serum (−), anti-gp150 (+), or anti-MHV-68 (+). Proteins were then analyzed by SDS-PAGE and Western blotting. Biotinylated proteins were detected by probing with streptavidin-peroxidase and enhanced chemiluminescence. The positions of molecular weight markers (in thousands) and gp150 are shown on the left.

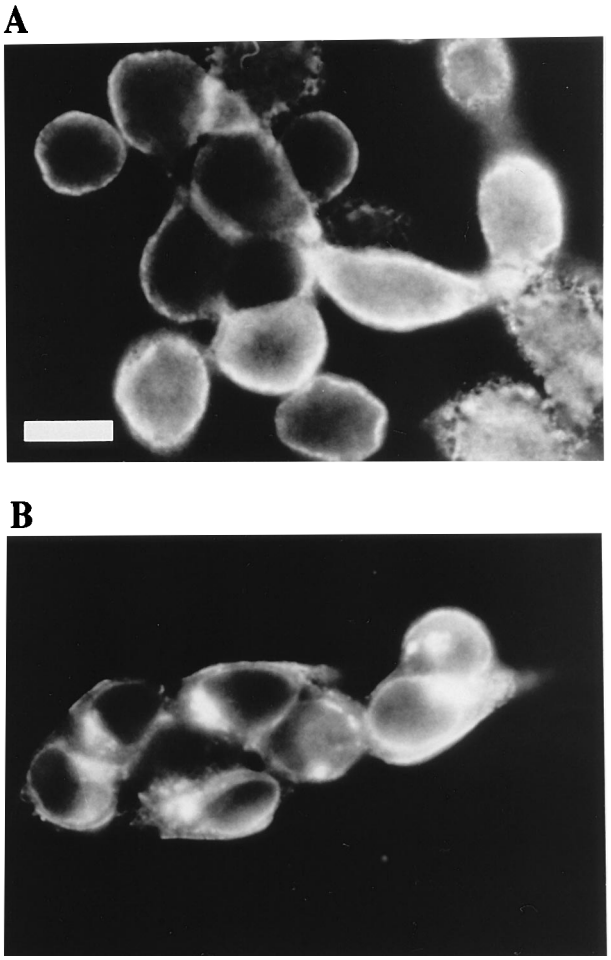

FIG. 7. Immunofluorescence analysis of MHV-68-infected cells. C127 cells were infected with 10 PFU of MHV-68 per cell for 18 h. Slides were then either left untreated (A) or fixed with acetone (B) before the immunofluorescence staining procedure. Cells were stained with anti-gp150, and immunofluorescence was then visualized with a UV microscope. The two panels are shown at the same magnification (size bar, 10 μm).

A number of cell surface-labelled proteins, including one with a molecular weight of 150,000, were detected when anti-MHV-68 serum was used as a positive control. Anti-gp150 immunoprecipitated biotinylated gp150 but not gp130 from the labelled cell surface. This result indicates that gp150 alone is reaching the infected-cell surface.

Taken together, these results demonstrate that gp130 is the precursor of gp150. For this reason, we named the protein product of BPRF1 gp150.

Localization of gp150. The localization of gp150 within infected cells was studied initially by immunofluorescence. Cells were grown on slides and then infected with MHV-68. They were then either fixed and permeabilized by using acetone prior to immunofluorescence analysis or stained live without being fixed.

Controls in which anti-gp150 was reacted with mock-infected cells and in which MHV-68-infected cells were probed with preimmune serum were entirely negative. In addition, no fluorescence was seen when live cells were reacted with anti-MHV gB, which is known to be absent from the infected-cell surface (32). Thus, antibody did not penetrate the cells during the processing of live cells. Figure 7A shows the results of probing live, unfixed, MHV-68-infected cells with anti-gp150. Fluorescence can be clearly seen localized to the cytoplasmic membrane, confirming that gp150 is expressed on the surface of infected cells. Since the antibody was raised to the predicted ectodomain of the protein, the staining of unfixed cells also confirms the orientation of the protein in the membrane. When similar cells were probed with anti-gp150 after being fixed and permeabilized with acetone (Fig. 7B), the fluorescence could be seen on the cytoplasmic membrane but also in the nuclear margin and as intense patches within the cytoplasm adjoining the nucleus.

To confirm this pattern of localization and to determine whether gp150 was a component of the virion, we performed immunoelectron microscopy. Infected and mock-infected cells were fixed for immunoelectron microscopy, embedded in low-temperature embedding resin, and cut into 60-nm sections. The sections were then probed with either anti-gp150 or control antibodies and anti-rabbit antibodies conjugated to 5-nm-diameter gold particles.

No significant labelling was obtained with mock-infected cells or with cells probed with preimmune serum. Figure 8 shows that at high magnification, anti-gp150 could be seen labelling the surface of MHV-68 particles which were released

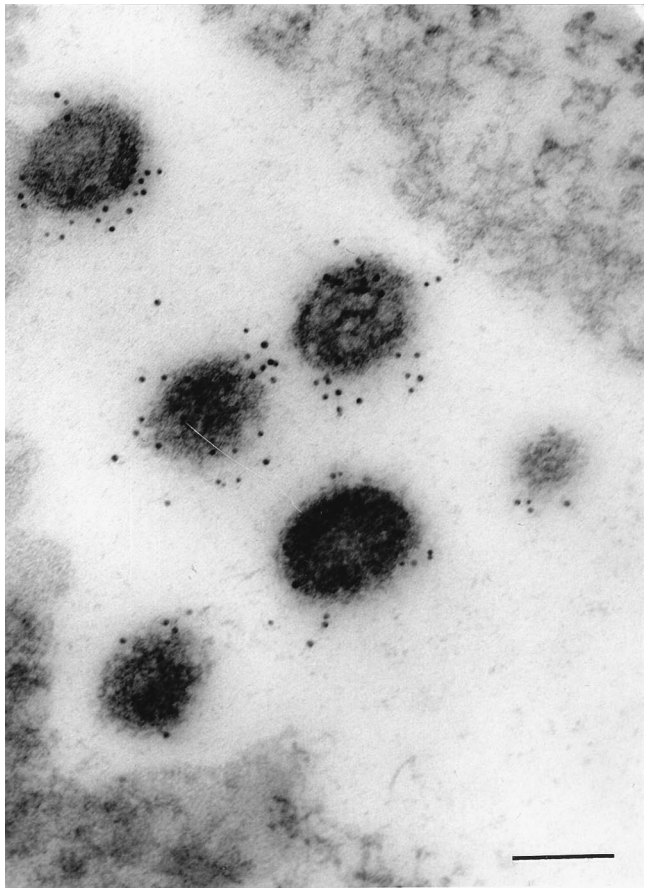

FIG. 8. Immunoelectron microscopy of MHV-68-infected cells. C127 cells were infected with 10 PFU of MHV-68 per cell for 18 h before being fixed, embedded in low-temperature resin, sectioned, and labelled with a combination of anti-gp150 antiserum and 5-nm-diameter colloidal gold. The sections were counterstained and analyzed by using a transmission electron microscope. A group of MHV-68 particles released from the cytoplasm at the surface of the cell is shown. Colloidal gold labelling indicates that gp150 was found at the surface of the virus particles. Size bar, 100 nm.

from the surface of infected cells. The distribution of the gold particles suggested that gp150 was a component of the virion membrane and that the protein protrudes from the surface of the particle.

Thus, gp150 was localized to the nuclear margins, in the cytoplasm, and on the surface of infected cells. The N-terminal domain was localized to the outside of infected cells and virion membranes, confirming that gp150 is a type I membrane glycoprotein.

Anti-gp150 antibodies neutralize virus infectivity. The data presented above have shown that MHV-68 gp150 was a component of the virion membrane. We therefore wished to assess the influence of gp150 on virus infectivity. To do this, we analyzed the ability of polyclonal anti-gp150 to neutralize MHV-68. The anti-gp150 had been raised against a portion of the molecule which comprised a significant portion of the ectodomain and therefore might contain neutralizing epitopes. We used this reagent in a conventional plaque reduction assay using preimmune serum as a background, anti-MHV-68 gB, which was known to be nonneutralizing (32), as a negative control, and polyclonal anti-MHV-68 as a positive control. The experiment was repeated on three occasions with comparable results. The results showed that anti-MHV-68 exhibited strong neutralizing activity with no plaques being observed at dilutions of serum down to 1/100, whereas the anti-MHV-68 gB exhibited no neutralizing activity. Anti-gp150 displayed weak neutralizing activity with a 50% reduction in plaque number occurring at a 1/30 dilution and an 84% reduction at a 1/10 dilution. These results showed that gp150 is a neutralizing determinant expressed by MHV-68.

DISCUSSION

We have identified, sequenced, and characterized a novel virion glycoprotein of MHV-68 referred to as gp150. The ORF (BPRF1) spans the junction of the *Bam*HI P_2 and Q fragments of the viral genome, and the predicted amino acid sequence showed limited homology with EBV gp340/220. A 1.5-kb cDNA corresponding to the gene was cloned and sequenced. Biochemical studies revealed that the product of this gene was a glycoprotein with two forms with molecular weights of 150,000 (gp150) and 130,000 (gp130). gp130 was a precursor form and contained only high-mannose, N-linked glycans; only gp150 reached the cell surface. gp150 was localized to the nuclear margins, patches in the cytoplasm, and the plasma membrane of infected cells as well as on the virion membrane. Finally, biological data showed that antibodies to gp150 mediated virus neutralization in the absence of complement.

In the gammaherpesviruses for which sequence data are available (herpesvirus saimiri, equine herpesvirus 2, BHV-4, and EBV), a potential glycoprotein gene has been found at the same locus as the gene for gp150 (1, 2, 20, 39). In EBV, there are a number of genes in the same region, including two glycoprotein genes, BZLF2 and the gp340/220 gene (2). Comparisons of the predicted amino acid sequence of gp150 with the positionally analogous glycoprotein sequences from the other gammaherpesviruses showed that the only product with which gp150 had any significant sequence homology was EBV gp340/220 (Table 1). Like gp340/220, MHV-68 gp150 also has a domain containing proline-rich repeats 22 amino acids in length situated in the ectodomain towards the membrane anchor (Fig. 1). The function of this domain is not known; however, its high proline content suggests that it may have little secondary structure and may act as a spacer between other functional domains. The EBV gp340/220 molecule is responsible for the binding of virus to B lymphocytes via the CD21 molecule and mediating the initial step of virus infection. The motif on gp340/220 responsible for this interaction is EDPGFFNVE, situated near the amino terminus of the molecule (23). However, there is no domain of similar sequence in the MHV-68 gp150. Human and murine CD21 molecules are extremely closely related. This suggests that although gp340/220 and gp150 may show evidence of being related via an ancestral gene, the MHV-68 molecule may differ in its ability to interact with CD21. However, like EBV, MHV-68 is tropic for B cells both in vitro and in vivo (36, 37). MHV-68 may therefore encode a molecule which interacts with a B-cell-specific marker. gp150 is expressed on the surface of the virion, and antibodies to gp150 neutralize virus infectivity in the absence of complement. This suggests that gp150 may be involved with either binding of the virus to a cellular receptor or a postbinding event involved with virus penetration. Further studies, such as inhibition of virus binding with antibody or recombinant protein as well as observation of the effect of deleting the gene, are required to determine in which of these mechanisms gp150 is involved and hence whether gp150 is functionally analogous to EBV gp340/220.

The predicted molecular weight of the core unglycosylated protein was 48,000. However, the apparent molecular weight of the final product was considerably greater. This could not be accounted for solely by the presence of carbohydrate, since the molecular weight of gp130 after digestion with endo-H, which leaves the core protein, was 125,000 (Fig. 4). In addition, in vitro transcription and translation of the gp150 cDNA produced two proteins, the larger of which had a molecular weight similar to that of the unglycosylated product. It seems likely, therefore, that the larger apparent molecular weight is due to the extremely high proline content (23%) of the protein, which is known to increase the apparent molecular weight on SDS-PAGE gels (15).

An additional, minor, unglycosylated product (p110) which was antigenically related to gp150 was also seen. Pulse-chase analysis (Fig. 3) showed that p110 was not the precursor of gp130 or gp150, and in vitro transcription and translation (Fig. 5) showed that p110 was translated from the same message as gp130. It seems likely, therefore, that p110 arises from the use of a downstream translational initiation site at nucleotide 792 (Fig. 1).

gp150 is the first MHV-68 virion-associated glycoprotein to be sequenced and studied. The only other MHV-68 protein to have been studied is the gB homolog, and this, like the EBV gB, is retained in the endoplasmic reticulum and not incorporated in the virion (32). In contrast to gB, gp150 is expressed on the surface of infected cells as well as being a neutralizing determinant on the surface of the virion. It is therefore a potential target for the immune response, in terms of not only virus-specific cytotoxic T cells but also naturally occurring neutralizing antibodies and antibody-dependent cell-mediated cytotoxicity. Thus, gp150 is a unique and valuable reagent for the future study of MHV-68 as a gammaherpesvirus model with respect to both its biology in vitro and the pathogenesis and immune response in vivo.

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