JOURNAL OF VIROLOGY, Dec. 1992, p. 7217–7224
0022-538X/92/127217-08$02.00/0

Vol. 66, No. 12

Characterization of a Vaccinia Virus-Encoded 42-Kilodalton Class I Membrane Glycoprotein Component of the Extracellular Virus Envelope

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Received 22 July 1992/Accepted 8 September 1992

Using a reverse genetic approach, we have demonstrated that the product of the B5R open reading frame (ORF), which has homology with members of the family of complement control proteins, is a membrane glycoprotein present in the extracellular enveloped (EEV) form of vaccinia virus but absent from the intracellular naked (INV) form. An antibody (C′-B5R) raised to a 15-amino-acid peptide from the translated B5R ORF reacted with a 42-kDa protein (gp42) found in vaccinia virus-infected cells and cesium chloride-banded EEV but not INV. Under nonreducing conditions, an 85-kDa component, possibly representing a hetero- or homodimeric form of gp42, was detected by both immunoprecipitation and Western immunoblot analysis. Metabolic labeling with [^{3}H]glucosamine and [^{3}H]palmitate revealed that the B5R product is glycosylated and acylated. The C-terminal transmembrane domain of the protein was identified by constructing a recombinant vaccinia virus that overexpressed a truncated, secreted form of the B5R ORF product. By N-terminal sequence analysis of this secreted protein, the site of signal peptide cleavage of gp42 was determined. A previously described monoclonal antibody (MAb 20) raised to EEV, which immunoprecipitated a protein with biochemical characteristics similar to those of wild-type gp42, reacted with the recombinant, secreted product of the B5R ORF. Immunofluorescence of wild-type vaccinia virus-infected cells by using either MAb 20 or C′-B5R revealed that the protein is expressed on the cell surface and within the cytoplasm. Immunogold labeling of EEV and INV with MAb 20 demonstrated that the protein was found exclusively on the EEV membrane.

Poxviruses, unlike other DNA viruses, transcribe and replicate their genomes and assemble progeny virions entirely within the cytoplasm of infected cells (for a review, see reference 20). As viewed by transmission electron microscopy, the first steps in virion morphogenesis involve the formation of spicule-coated membranes that enclose a granular matrix (5). Further maturation results in an electron-dense core structure. These mature particles, called intracellular naked virions (INV), are infectious when released from lysed cells. Some of the INV become enwrapped by a double-layer cisternal membrane and move to the cell periphery (12, 19, 25, 26). There, fusion of the outermost viral membrane with the plasma membrane occurs. Those virions that are released into the medium are referred to as the extracellular enveloped virions (EEV). The EEV form of vaccinia virus retains an outer membrane not present on INV. EEV are thought to be important in the long-range dissemination of virus within an animal and thereby contribute to virulence (4, 23). In fact, immunity to the EEV envelope proteins is an important protective mechanism (1, 23, 35). The amount of vaccinia virus released as EEV varies among virus strains and cell types (22, 23). Some of the wrapped virus remains attached to the outside of the cell and has been called cell-associated enveloped virus (2). Cell-associated enveloped virus appears to be important in the spread of the virus to neighboring cells, especially in vaccinia virus strains that make little EEV (3). Identification and characterization of the proteins found in the outer envelope of vaccinia virus may lead to a better understanding of the processes involved in virus assembly and dissemination.

The outer envelope of EEV was reported to contain nine glycosylated proteins and one nonglycosylated protein (10, 21, 22). Recent biochemical characterization of purified EEV by using monoclonal antibodies (MAbs) suggested, however, that these nine glycoproteins might represent the products of a smaller number of genes (24). MAbs that recognized the 89-kDa hemagglutinin glycoprotein also bound to smaller proteins of 32, 41.5, 52, and 76 kDa as well as to a larger 220-kDa complex. Similarly, by using another panel of MAbs, a group of proteins with molecular masses of 23 to 28 kDa was shown to have a common protein backbone. A third glycoprotein identified by MAbs raised to EEV has an apparent molecular mass of 42 kDa and is here called gp42.

Efforts are being made to identify the genes encoding the EEV-specific proteins. Thus, the nonglycosylated protein P37 was mapped to the F13L open reading frame (ORF) (11), and the hemagglutinin was mapped to the A56R ORF (31). Several candidate membrane proteins were predicted from the hydrophobic regions and potential N-glycosylation sites in the translated DNA sequences (9, 33). By preparing antibody to some of these proteins, Duncan and Smith (7) and Engelstad et al. (8) provided evidence that the *Sal*L4R ORF and the B5R ORF encode 22- to 24-kDa and 42-kDa EEV glycoproteins, respectively. The protein encoded by the B5R ORF is of additional interest since it has similarities to members of the family of complement control proteins. In

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fact, B5R most closely resembles the product of the vaccinia virus C21L ORF (16) (in Copenhagen sequence nomenclature it is called C3L) (9), which encodes the vaccinia virus complement control protein (VCP). VCP, however, has no transmembrane regions and is a major secreted protein from vaccinia virus-infected cells. Furthermore, VCP can inhibit complement-mediated lysis of sensitized erythrocytes and complement-enhanced neutralization of vaccinia virions, suggesting that it has a role in defense against the host immune system (14, 15).

We have taken a reverse genetic approach similar to that of Smith and coworkers (7, 8) and in this report provide direct proof that the B5R ORF encodes the 42-kDa glycoprotein described by Payne (24). In addition, the signal and transmembrane peptides were identified, and the 42-kDa protein was localized within the infected cell and EEV by immunofluorescence and electron microscopy, respectively.

MATERIALS AND METHODS

Cells and viruses. HeLa cell monolayers were maintained in Dulbecco's medium (Quality Biological, Inc., Gaithersburg, Md.) containing 10% fetal bovine serum (FBS). TK^- 143 cells were maintained in minimal essential medium (MEM) containing 10% FBS and 25 μg of bromodeoxyuridine per ml. BSC-1 and RK_{13} cells were maintained in MEM containing 10% FBS. Cells were typically infected with a crude vaccinia virus stock (strain WR [ATCC Vr119] or IHD-J, obtained from S. Dales, University of Western Ontario, London, Ontario, Canada) at a multiplicity of infection of 5 to 10 PFU per cell in medium containing 2.5% FBS. Infected cells were incubated at 37°C in a 5% CO_2 atmosphere.

INV and EEV forms of vaccinia virus (strain IHD-J) were sedimented through a 36% sucrose cushion, banded on a cesium chloride gradient as previously described (22), and repelleted through a 36% sucrose cushion. To extract envelope proteins, approximately 20 μg of virus (1 optical density unit measured at 260 nm equals 64 μg) was treated with 0.5% Nonidet P-40 at room temperature for 15 min.

Infected RK_{13} cells were metabolically labeled with 30 μCi of [^{35}S]methionine per ml, 30 μCi of [^{3}H]glucosamine per ml, and 50 μCi of [^{3}H]palmitate per ml as described previously (24) except that the medium was serum free.

Antibodies. Polyclonal rabbit antibody C′-B5R was raised to the peptide sequence CSLDVVQYEQEIESLE (residues 261 to 275). The underlined amino acids span an area preceding the large hydrophobic domain of the translated B5R ORF (see Fig. 1). A cysteine was included at the N terminus to facilitate conjugation of the peptide to keyhole limpet hemocyanin by using an Imject Activated Immunogen Conjugation Kit (Pierce) as described by the manufacturer. After a primary injection and two boosts with antigen, the rabbit was bled to prepare antiserum. MAb 20 has been described previously (24). For indirect immunofluorescence, fluorescein isothiocyanate-conjugated goat anti-rabbit immunoglobulin G (Boehringer Mannheim) and rhodamine-conjugated rabbit anti-mouse immunoglobulin G (Dako Corp.) were used. For immunogold electron microscopy studies, protein A-conjugated 20-nm colloidal gold particles (Polysciences, Inc.) were used.

Construction of a recombinant vaccinia virus expressing a truncated soluble form of gp42. As detailed below, a segment of the B5R ORF was amplified by the polymerase chain reaction (PCR) and ligated to a synthetic strong late vaccinia virus promoter in the transfer vector pMJ602 (6). The vector pMJ602 also contains the *Escherichia coli lacZ* gene downstream of a vaccinia virus promoter as well as flanking vaccinia virus *tk* sequences to permit homologous recombination. Oligonucleotide primers 5′-TTT AAG CTT ATG AAA ACG ATT TCC GTT GTT-3′ and 5′-TTT GCT AGC CTA TTA TTC TAA CGA TTC TAT TTC TTG-3′, based on the Copenhagen sequence of vaccinia virus and containing restriction sites *Hin*dIII and *Nhe*I (underlined), were used to amplify a 820-bp segment of the B5R ORF from a plasmid containing the *Hin*dIII-*Bam*HI segment of the *Hin*dIII B fragment of vaccinia virus (strain WR) DNA. The PCR conditions were 15 cycles of denaturation at 94°C for 2 min, annealing at 55°C for 2 min, and extension at 72°C for 2 min 10 s. After amplification, the PCR product was gel purified. The PCR product and pMJ602 were digested with restriction enzymes *Hin*dIII and *Nhe*I (Bethesda Research Laboratories) and ligated together with T4 DNA ligase (Bethesda Research Laboratories). The synthetic promoter region and the entire insert of the resulting plasmid (pSI-77P_2) were sequenced to confirm the DNA structure. CV-1 cells were infected with vSIGK-8, a previously described recombinant vaccinia virus that does not synthesize the protein encoded by the C3L ORF (13), and then transfected with pSI-77P_2. The progeny virus was harvested, and recombinant vSI-13 was isolated on TK^- 143 cells by selecting virus plaques that were resistant to bromodeoxyuridine and stained blue in the presence of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal).

Immunoprecipitations and Western immunoblot analysis. Infected cell lysates were clarified by ultracentrifugation at 30,000 rpm for 60 min in a Ti42.2 rotor (Beckman), and immunoprecipitations were carried out in radioimmunoprecipitation assay buffer as previously described (24). The antigen-antibody complexes were bound to protein A beads; after the beads were washed, the proteins were eluted by boiling for 5 min in 2% sodium dodecyl sulfate (SDS)–60 mM Tris-HCl (pH 6.8)–10% glycerol in the presence or absence of 50 mM dithiothreitol (DTT). The released proteins were applied to a polyacrylamide–0.1% SDS gel with stacking and separating portions containing 5 and 10% polyacrylamide, respectively. For Western blotting, the resolved protein bands were electrophoretically transferred onto nitrocellulose, using a Genie Electrophoretic Blotter (Idea Scientific Co., Minneapolis, Minn.). The blots were then incubated in 2% bovine serum albumin (BSA) in phosphate-buffered saline (PBS)–0.2% Tween and then with a 1:500 dilution of the indicated antiserum in PBS-Tween. After being washed with PBS-Tween, the nitrocellulose was incubated with 0.1 μCi of [^{125}I]protein A (Amersham) per ml in PBS-Tween, washed again, and subjected to autoradiographic analysis.

N-terminal sequence. A 25-cm^2 flask containing a confluent monolayer of BSC-1 cells was infected with vSI-13 at a multiplicity of 5 PFU per cell in 1 ml of serum-free Opti-MEM (GIBCO). After a 2-h absorption period at 37°C in a 5% CO_2 atmosphere, the inoculum was removed and 3 ml of fresh serum-free Opti-MEM was added. Incubation was continued for 48 h, at which time the medium was removed and EEV was pelleted by centrifugation at maximal speed in a microcentrifuge for 30 min at 4°C. The supernatant was then concentrated in a Centricon-10 (Amicon, Beverly, Mass.) to a volume of about 120 μl. Of this material, 24 μl was loaded on a 4% stacking–10% separating polyacrylamide–SDS gel run in Tricine buffer (29). After electrotransfer onto a polyvinylidene difluoride membrane (Bio-Rad), a band of the correct molecular weight was visualized by using Ponceau C stain. The band was cut out, and the N-terminal

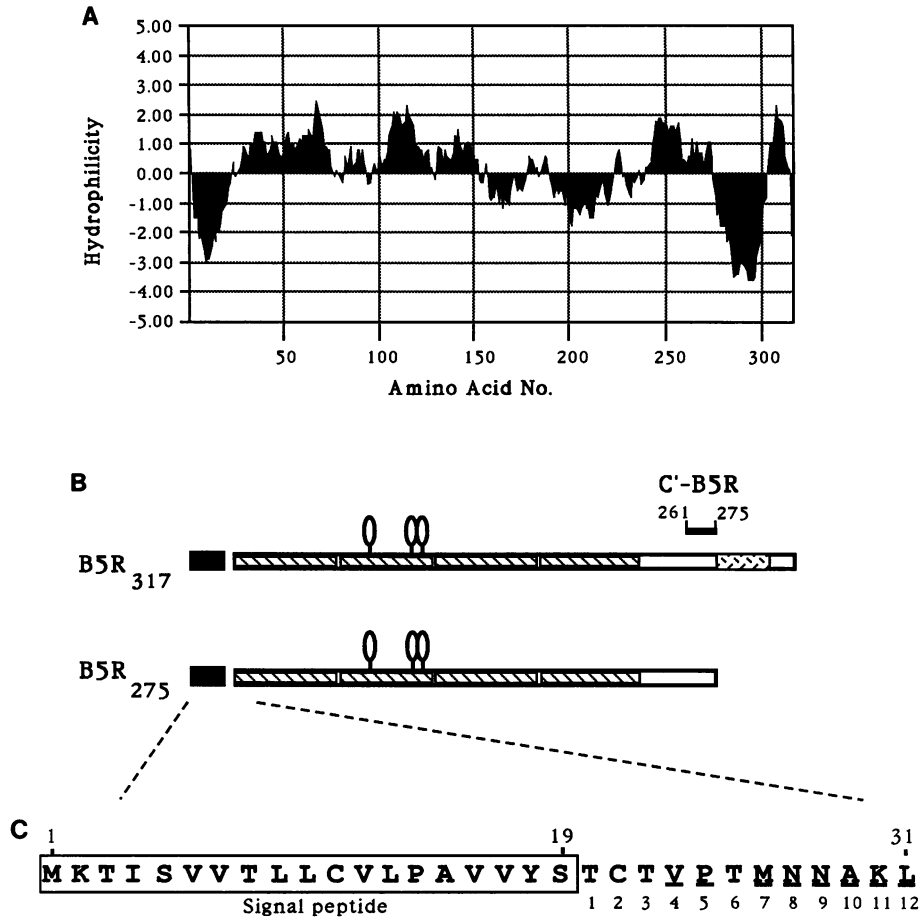

FIG. 1. Analysis of the B5R ORF. (A) Hydrophilicity plot of the B5R ORF. (B) Diagram of the protein encoded by the full-length 317-amino-acid B5R ORF ($B5R_{317}$). ■, putative signal peptide; ▨, 60-amino-acid repeats present in complement control proteins; ▭, potential membrane anchor; ⚲, a potential N-linked glycosylation site. $B5R_{275}$ represents the recombinant protein encoded by vSI-13, in which the carboxy-terminal hydrophobic domain was removed. The bar from amino acids 261 to 275 represents the 15-amino-acid peptide used to immunize rabbits to generate a polyclonal antibody (C'-B5R). (C) The first 32 amino acids from the deduced sequence of the B5R ORF. The numbers 1 to 12 under the sequence refer to the Edman degradation cycle, and the underlined amino acids are those unambiguously identified by the protein sequence of the soluble form of gp42. Cysteine and threonine residues could not be determined because of the sequencing methodology. The signal peptide is boxed.

sequence of the protein was obtained by using a model 120A phenylthiohydantoin (PTH) analyzer (Applied Biosystems, Inc., Foster City, Calif.) according to the manufacturer's program, NORMAL-1. Reverse-phase high-pressure liquid chromatography analysis of the PTH amino acids was done by using a Brownlee PTH-C_{18} column (2.1 by 220 mm) (18).

Immunofluorescence and electron microscopy. HeLa monolayer cells, grown on coverslips, were infected with 5 PFU of vaccinia virus (WR strain) per cell. At 8 h after infection, cells were washed twice in PBS, fixed in 3% paraformaldehyde for 20 min at room temperature, and then washed again in PBS. Permeabilization and subsequent incubation and washing steps were carried out in PBS–0.05% saponin (Calbiochem); otherwise, PBS was used without detergent. Cells were incubated for 30 min with antibody at the indicated dilutions, washed three times, and then incubated with either fluorescein isothiocyanate-conjugated goat anti-rabbit antibody or rhodamine-conjugated rabbit anti-mouse antibody for 30 min at room temperature. Cells were washed three times and then rinsed twice with H_2O, and the coverslips were mounted on slides in 5% Dabco (Sigma Chemical Co.) in Mowiol (Hoechst AG; Calbiochem). Stained cells were viewed with a fluorescence microscope.

Carbon-coated copper mesh grids (kindly provided by M. Misra, Laboratory of Structural Biology, National Institute of Arthritis and Musculoskeletal and Skin Diseases) were floated on CsCl-purified EEV or INV suspensions and then washed with PBS and Tris-buffered saline (TBS; 25 mM Tris [pH 8.2], 3 mM KCl, 137 mM NaCl) for 2 min. The virus-coated grids were incubated in TBG (TBS [pH 8.2], 0.1% BSA, 1% gelatin) for 15 min and then in a 1/50 dilution of MAb 20 in TBS–1% BSA for 1 h. Grids were washed for 10 min in TBG, and bound antibody was detected by incubation with colloidal gold-conjugated protein A diluted 1/10 in TBS–1% BSA. Grids were washed for 5 min with TBG and then TBS, and proteins were fixed in 2% glutaraldehyde (Polysciences, Inc.)-TBS. Finally, viral particles were negatively stained with 1% uranyl acetate.

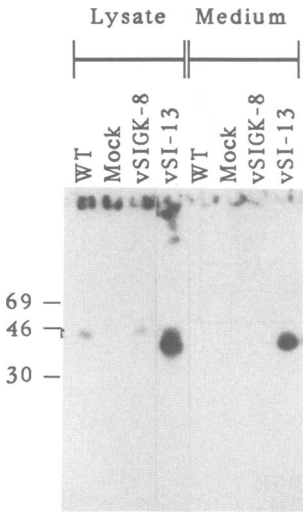

FIG. 2. Western blot analysis of cell lysates and media from cells infected with wild-type and recombinant vaccinia viruses. Individual wells of a six-well plate containing RK_{13} cells were infected with wild-type vaccinia virus strain WR (WT), mutant vSIGK-8, or mutant vSI-13 at 5 PFU per cell or were mock infected in serum-free Opti-MEM for 24 h. The media were removed, and any EEV was pelleted by high-speed centrifugation. Proteins in the clarified media were precipitated at 4°C in 10% trichloroacetic acid. The protein pellets were washed with 70% ethanol, dried, and resuspended in 75 μl of 60 mM Tris (pH 6.8). The cell monolayers were lysed with 450 μl of 0.5% Triton X-100–20 mM Tris (pH 7.0) in PBS. To 15 μl of the protein isolated from the media or the cell lysates, 5× Laemmli loading buffer (17) and 2 μl of 2-mercaptoethanol were added. After boiling, samples were subjected to SDS-PAGE and electrophoretically transferred to nitrocellulose. Antiserum C′-B5R at a final concentration of 1:500 was used to probe the blot, which was then incubated with ^{125}I-labeled staphylococcal protein A. An autoradiogram is shown. Numbers on the left refer to the molecular masses of ^{14}C-labeled protein markers (Bethesda Research Laboratories) in kilodaltons.

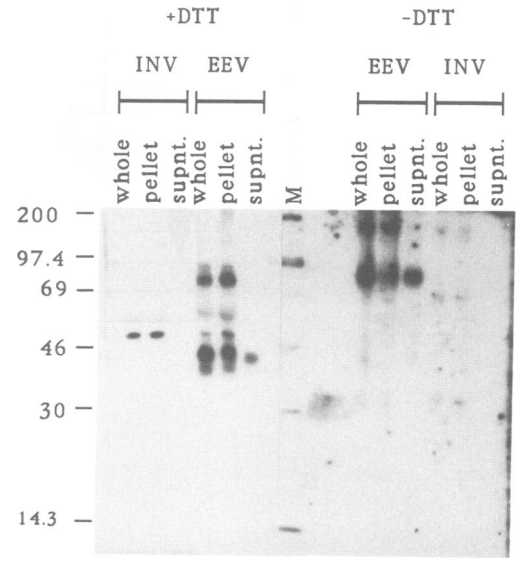

FIG. 3. Western blot analysis of INV and EEV proteins. Equal amounts of CsCl-banded INV and EEV from vaccinia virus (strain IHD-J) were incubated in 0.5% Nonidet P-40 at room temperature and centrifuged to obtain pellet and supernatant (supnt.) fractions. The proteins were boiled in Laemmli loading buffer in the presence (+DTT) or absence (−DTT) of reducing agent and analyzed by SDS-PAGE. The proteins were transferred to nitrocellulose and incubated with antiserum C′-B5R at a final concentration of 1:500 followed by ^{125}I-labeled staphylococcal protein A. Molecular weights (in thousands) based on ^{14}C-labeled protein markers (lane M) are indicated.

RESULTS

Expression of a truncated form of the B5R ORF. The nucleotide sequences of the B5R ORF from the Copenhagen (9) and WR (33) strains of vaccinia virus indicated only four amino acid differences. A hydrophobicity plot of the translated ORF is consistent with the presence of N- and C-terminal hydrophobic domains that could serve as a signal peptide and a membrane anchor, respectively (7, 9, 33). In addition, there are four tandem segments that resemble the 60-amino-acid repeats found in complement-binding proteins and three putative N-linked glycosylation sites within the predicted extracellular domain (9, 33). We decided to test the deductions regarding the signal and anchor sequences by constructing a recombinant vaccinia virus that would express a truncated form of the B5R ORF, lacking the C-terminal hydrophobic domain (Fig. 1). Since the product of the C3L ORF is a secreted protein with homology to B5R, we used vSIGK-8, a C3L deletion mutant (13), as the parental virus. A truncated form of B5R (amino acids 1 through 275) regulated by a strong synthetic late viral promoter was inserted into the *tk* locus of vSIGK-8. The resulting recombinant vaccinia virus, vSI-13, was expected to overexpress a truncated form of B5R as well as normal amounts of the complete B5R product but not the C3L product. We anticipated that if the deleted domain acted as the only membrane anchor, vSI-13-infected cells would secrete a truncated soluble form of the B5R ORF product into the medium. Using the polyclonal antibody C′-B5R raised against a 15-amino-acid peptide deduced from the B5R ORF sequence (Fig. 1B), we identified a soluble 41-kDa protein in the medium of vSI-13-infected cells but not in the medium of wild-type- or vSIGK-8-infected cells (Fig. 2). A protein with an apparent molecular mass of 42 kDa was present in the lysates of cells infected with all three viruses and represents the cell-associated, full-length product of the B5R ORF (Fig. 2). In addition, the lysate from vSI-13-infected cells contained a large amount of the truncated protein (Fig. 2). These results provided evidence that the full-length protein is membrane anchored by the C-terminal hydrophobic domain and can be classified as a type I membrane protein.

Identification of the signal peptide. The clarified, serum-free medium from cells infected with vSI-13 was concentrated, and the secreted 41-kDa protein was purified by SDS-polyacrylamide gel electrophoresis (PAGE). The N-terminal sequence of the protein secreted from vSI-13-infected cells was determined by Edman degradation. The sequence could be aligned with that predicted from the B5R ORF and revealed that the signal peptide of the mature protein was cleaved at the 19th amino acid after the initiating methionine (Fig. 1C).

The B5R ORF encodes a protein found in the EEV form of vaccinia virus. We were interested in further characterizing and localizing the protein encoded by the membrane-anchored form of the B5R ORF. The INV and EEV forms of vaccinia virus (strain IHD-J) were purified by CsCl gradient centrifugation, and their proteins were dissociated with SDS and separated by electrophoresis. By Western blot analysis,

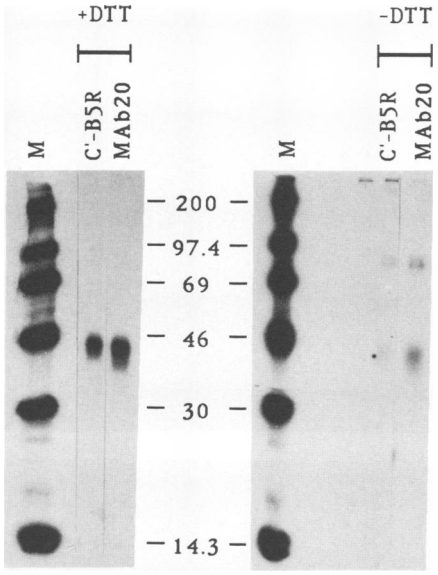

FIG. 4. Immunoprecipitation of proteins from infected cells metabolically labeled with [^{35}S]methionine. Cells infected with vaccinia virus strain IHD-J were metabolically labeled with [^{35}S]methionine. Cell lysates were immunoprecipitated with either antiserum C'-B5R or MAb 20, and the protein samples were boiled in SDS in the presence (+DTT) or absence (−DTT) of reducing agent. Molecular weights (in thousands) based on ^{14}C-labeled protein markers (lanes M) are indicated.

antibody C'-B5R reacted with 42- and 85-kDa proteins found in CsCl-banded EEV which were not present in CsCl-banded INV (Fig. 3). A minor 50-kDa band was detected in both INV and EEV. The 42-kDa protein was only partially extracted from EEV with 0.5% Nonidet P-40, indicating a tight association with the particles.

The high-molecular-mass band of approximately 85 kDa was more abundant when samples were not treated with DTT prior to electrophoresis, suggesting that this band is a disulfide-bonded homo- or heterodimeric form of the B5R protein. The nature of the minor 50-kDa band, possibly a cross-reacting species, was not determined.

The apparent molecular weight and location of the protein in EEV suggested that the B5R ORF encoded the protein gp42, which was shown by Payne (24) to react with a monoclonal antibody (MAb 20) raised to the EEV form of vaccinia virus. Immunoprecipitation of lysates of vaccinia virus-infected cells that had been metabolically labeled with [^{35}S]methionine was carried out to test this possibility. Identical gel patterns were obtained with use of either MAb 20 or C'-B5R (Fig. 4). Usually, the higher-molecular-weight form of the B5R protein was not detected in infected cell lysates unless reducing agent was omitted during sample preparation. Sometimes a weaker signal was seen on autoradiograms of samples run in the absence of reducing agent, suggesting that less protein had eluted from the protein A beads or had entered the gel.

Further evidence for the correspondence of the B5R product with gp42 was obtained by metabolically labeling infected cells with [^{3}H]glucosamine and [^{3}H]palmitate and immunoprecipitating them with MAb 20 or C'-B5R (Fig. 5A and B). Both the MAb raised to EEV and the antibody raised to a peptide within the B5R ORF immunoprecipitated a 42-kDa protein that was both glycosylated and acylated.

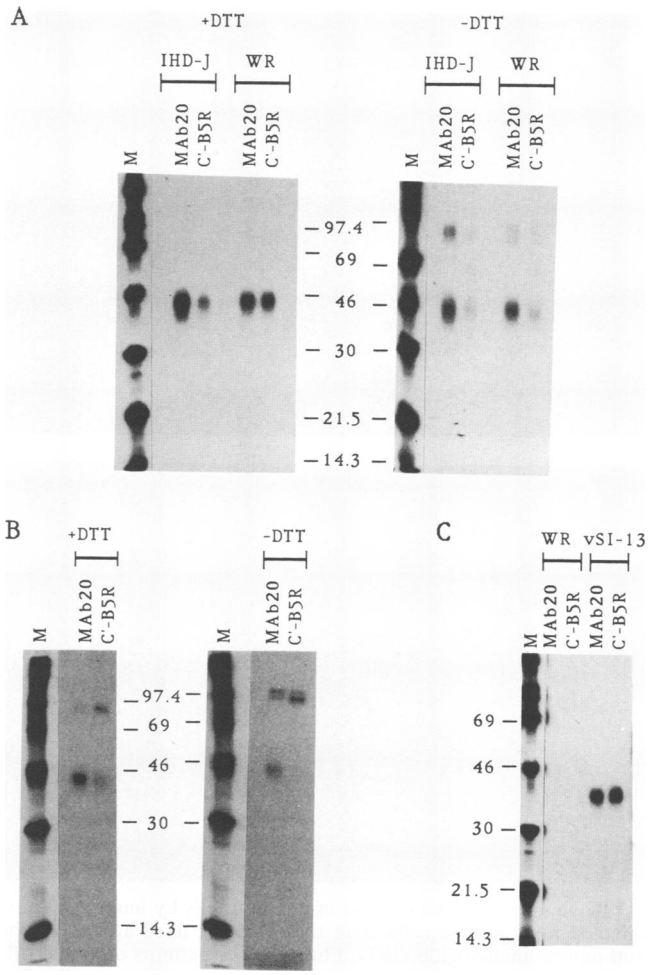

FIG. 5. Immunoprecipitation of proteins from infected cells metabolically labeled with [^{3}H]glucosamine and [^{3}H]palmitate. Cells infected with either wild-type vaccinia virus strain IHD-J or WR or with mutant vSI-13 were metabolically labeled with [^{3}H]glucosamine and [^{3}H]palmitate. Cell lysates were immunoprecipitated with either antiserum C'-B5R or MAb 20, and the protein samples were boiled in SDS in the presence (+DTT) or absence (−DTT) of reducing agent. Molecular weights (in thousands) based on ^{14}C-labeled protein markers (lanes M) are indicated. (A) Autoradiogram of immunoprecipitates from lysates of cells infected with strains IHD-J and WR metabolically labeled with [^{3}H]glucosamine; (B) autoradiogram of immunoprecipitates from lysates of cells infected with strain IHD-J metabolically labeled with [^{3}H]palmitate; (C) autoradiogram of immunoprecipitates of media from cells infected with either strain WR or vSI-13 metabolically labeled with [^{3}H]glucosamine.

When immunoprecipitates were not treated with DTT, the higher-molecular-weight species was observed. In certain instances (Fig. 4 and 5B), this material resolved on SDS-PAGE as a doublet of bands of approximately 80 to 83 and 85 to 88 kDa, as also noted by Engelstad et al. (8). There appeared to be no differences between the B5R protein metabolically labeled in cells infected with strain WR or IHD-J.

Direct proof that the B5R ORF encodes the 42-kDa protein identified by Payne (24) was obtained by using the recombinant virus vSI-13. Both MAb 20 and C'-B5R immunoprecipitated the truncated secreted form of gp42 from the

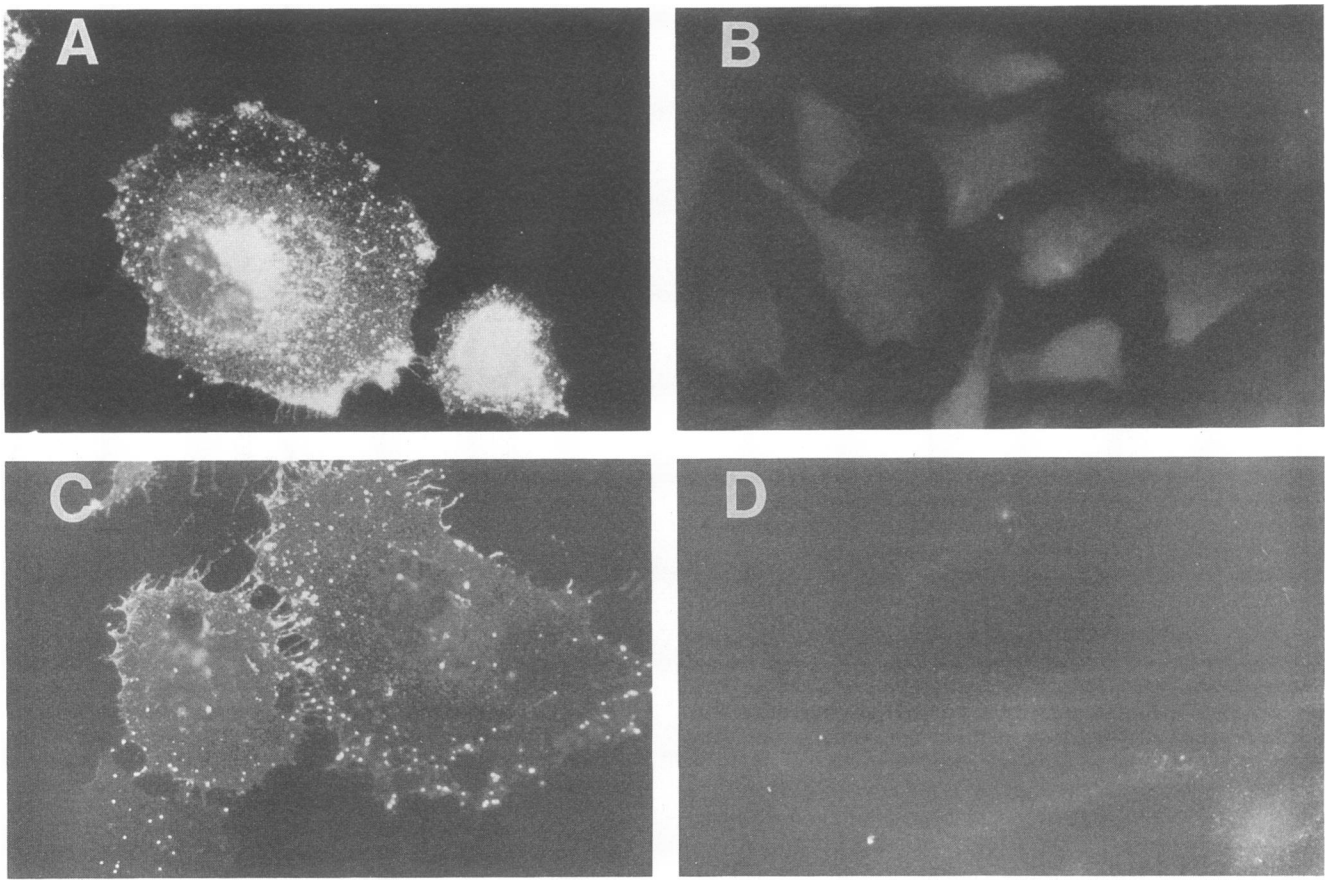

FIG. 6. Localization of gp42 in infected cells by immunofluorescence. HeLa cells were infected with vaccinia virus strain WR or mock infected for 8 h. The cells were then fixed or fixed and permeabilized and were stained with MAb 20 and rhodamine-conjugated rabbit anti-mouse immunoglobulin G. Fluorescence patterns of permeabilized infected cells (A), permeabilized uninfected cells (B), nonpermeabilized infected cells (C), and nonpermeabilized uninfected cells (D) are shown.

media of cells metabolically labeled with [^{3}H]glucosamine during infection with vSI-13 but not from the media of cells infected with wild-type virus (Fig. 5C). We also demonstrated that the secreted protein immunoprecipitated by MAb 20 reacted on a Western blot with the polyclonal antibody raised to the peptide from the B5R ORF (data not shown).

Localization of gp42 in infected cells. Indirect immunofluorescence was carried out by permeabilizing HeLa cells that had been infected with vaccinia virus and incubating them with MAb 20 followed by rhodamine-conjugated rabbit anti-mouse antibody. There was strong perinuclear staining, suggesting Golgi complex localization (Fig. 6A). Peripheral punctate staining that could represent virions also was seen. The punctate staining, as well as a more diffuse surface-staining pattern, was also evident on the surface of nonpermeabilized cells (Fig. 6C). Immunofluorescence with C′-B5R followed by fluorescein isothiocyanate-conjugated goat anti-rabbit antibody gave similar results (data not shown).

Localization of gp42 on EEV. Cesium chloride-banded INV and EEV were incubated with MAb 20 followed by colloidal gold-conjugated protein A. Electron microscopic examination revealed surface labeling of EEV but not INV (Fig. 7). The reverse result, labeling of INV but not EEV, was obtained when MAb C3 to the 14-kDa INV surface protein (28) was used (data not shown).

DISCUSSION

The presence of N- and C-terminal hydrophobic regions in the protein predicted to be encoded by the B5R ORF of vaccinia virus led us and Engelstad et al. (8) to investigate whether this protein might be associated with viral membranes. Engelstad et al. (8) reported that B5R is expressed early and late in infection and that the product is present in EEV but not INV. In addition, the protein was unstable when synthesized in the presence of tunicamycin, suggesting that it might be a glycoprotein. Our studies agree with and extend the conclusions of Engelstad et al. (8). Metabolic labeling experiments directly demonstrated that the B5R product is glycosylated and acylated and reacts with MAbs to gp42, an EEV protein. Deletion of the C-terminal hydrophobic domain resulted in the secretion of the truncated, glycosylated protein into the medium of infected cells. N-terminal sequencing of the secreted protein indicated that the signal peptide is cleaved, resulting in a processed protein that is 19 amino acids shorter. Immunofluorescence studies were consistent with Golgi complex and surface localization of the membrane-anchored protein. Finally, the B5R protein was shown by immunogold electron microscopy to be absent from INV and to be associated with the EEV membrane. Thus, the B5R protein is the third glycoprotein component of the EEV envelope to be identified genetically. The other

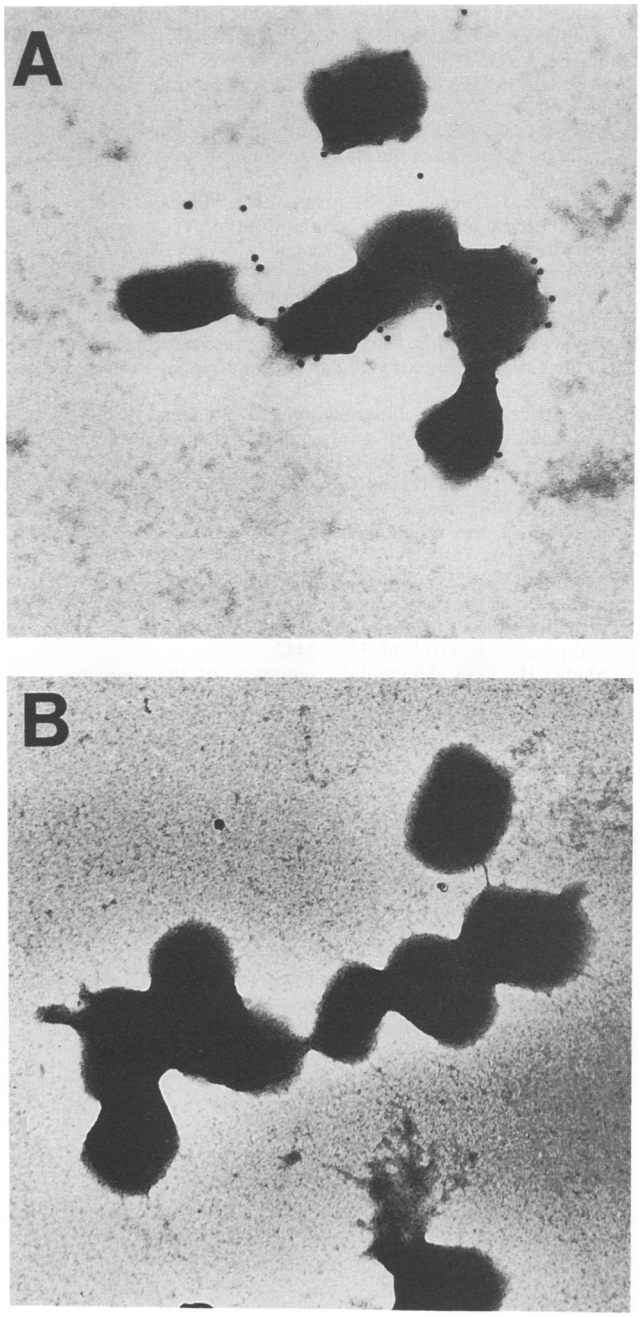

FIG. 7. Immunogold labeling of INV and EEV. CsCl-purified EEV (A) and INV (B) were incubated with MAb 20, and bound antibody was visualized with colloidal gold (20-nm particles) conjugated to protein A. Virus particles were negatively stained with uranyl acetate, and the staining patterns were examined by electron microscopy. Colloidal gold labeling of EEV was not observed when incubation with MAb 20 was omitted. Magnification, ×17,800.

EEV glycoproteins are the hemagglutinin (27) and the product of the *Sal*L4R ORF (7). Additionally, a nonglycosylated protein, P37, is specifically associated with EEV (10, 21).

The B5R protein, gp42, is a typical class I membrane glycoprotein with a cleavable N-terminal signal peptide and a C-terminal hydrophobic anchor domain. Although there is an additional hydrophobic region between amino acids 188 and 219, it is apparently not a transmembrane domain since a truncation at amino acid 275 led to secretion. Many membrane glycoproteins exist as dimers, trimers, and tetramers. When EEV was treated with SDS and DTT, both a 42-kDa protein and a higher-molecular-mass protein of approximately 85 kDa were resolved by SDS-PAGE. Omission of DTT resulted in a large increase in the proportion of the 85-kDa species. When samples were not reduced, the 85-kDa species could also be detected in infected cell extracts. The higher-molecular-mass forms of the B5R protein could represent gp42 homodimers or, alternatively, heterodimers between gp42 and another viral protein. The possibility that the 85-kDa species is formed by oxidation during solubilization has not been excluded. The vaccinia virus hemagglutinin is a class I glycoprotein and is both N and O glycosylated (32, 36). The product of *Sal*L4R ORF is a class II glycoprotein (7, 33). The latter protein is expressed late in infection and has homology to C-type animal lectins.

Genetic studies have shown that both P37 (2, 30) and the 23- to 28-kDa (7) glycoproteins are required for wrapping of INV and EEV formation. A frameshift mutation in the B5R ORF of an isolate of the Lister strain of vaccinia virus caused a small-plaque phenotype and host range restriction (34). The formation of small plaques, at least in some cell lines, suggests that B5R plays a role in virus transmission. Further analyses of this and other B5R mutants may provide further information on the role of the protein in the formation of the EEV envelope.

The homology of the B5R protein with complement control proteins has been previously noted (9, 33). The location of the protein on the surface of EEV would be consistent with it having a role in defense against the host immune system. Alternatively, the homology may reflect a role of the B5R protein in binding to a viral receptor for cell entry. The overexpression by recombinant virus vSI-13 of a secreted form of B5R with all four of the complement consensus repeats should facilitate binding studies for testing both hypotheses.

ACKNOWLEDGMENTS

We thank Rafael Blasco and Bob Doms for helpful discussions and critical review of the manuscript. Norman Cooper provided tissue culture cells, and Mark Garfield (Biological Resources Branch, NIAID) performed the N-terminal sequencing. We also thank Manoj Misra and Alasdair Steven of the Laboratory of Structural Biology (NIAMSD) for help with the electron microscopy.

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