The Vaccinia Virus B5 Protein Requires A34 for Efficient Intracellular Trafficking from the Endoplasmic Reticulum to the Site of Wrapping and Incorporation into Progeny Virions[†]

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The glycoproteins encoded by the vaccinia virus A34R and B5R genes are involved in intracellular envelope virus formation and are highly conserved among orthopoxviruses. A recombinant virus that has the A34R gene deleted and the B5R gene replaced with a B5R gene fused to the enhanced green fluorescent protein (B5R-GFP) gene was created (vB5R-GFP/ΔA34R) to investigate the role of A34 during virion morphogenesis. Cells infected with vB5R-GFP/ΔA34R displayed GFP fluorescence throughout the cytoplasm, which differed markedly from that seen in cells infected with a normal B5R-GFP-expressing virus (vB5R-GFP). Immunofluorescence and subcellular fractionation demonstrated that B5-GFP localizes with the endoplasmic reticulum in the absence of A34. Expression of either full-length A34 or a construct consisting of the luminal and transmembrane domains restored normal trafficking of B5-GFP to the site of wrapping in the juxtanuclear region. Coimmunoprecipitation studies confirmed that B5 and A34 interact through their luminal domains, and further analysis revealed that in the absence of A34, B5 is not efficiently incorporated into virions released from the cell.

Vaccinia virus is the prototype orthopoxvirus that was used in the global eradication of smallpox. During infection, orthopoxviruses produce multiple, morphologically distinct forms of progeny virions, starting with intracellular mature virus (IMV) (25). Although IMV are fully infectious, a subset of them are further modified by intracellular envelopment by the *trans*-Golgi or early endosomes and are called intracellular enveloped viruses (IEV) (34, 37). IEV are transported along microtubules to the cell periphery, where the outermost membrane of the envelope fuses with the plasma membrane, releasing cell-associated envelope virus (CEV) onto the surface of the cell (10, 14, 30, 42, 43). Some CEV are released from the cell and are called extracellular enveloped viruses (EEV). EEV and CEV are required for cell-to-cell spread and plaque formation on cell monolayers (1, 28).

Seven proteins encoded by the virus, namely, A33 (31), A34 (6), A36 (39), A56 (36), B5 (7, 17), F12 (38), and F13 (13), are unique to enveloped forms (IEV/EEV/CEV) of the virus (EV). With the exception of A56R, deletion of any one of the corresponding genes causes a reduction in plaque size on cell monolayers (2, 6, 7, 17, 26, 32, 48). Vaccinia virus open reading frames are designated by a capital letter indicating a HindIII restriction endonuclease fragment, a number indicating the position in the HindIII fragment, and a letter (L or R) indicating the direction of transcription, e.g., A34R. The corresponding protein is designated by a capital letter and number, e.g., A34. Both F13 (2) and B5 (8, 45) are thought to have important roles in intracellular envelopment because deletion of either gene has the greatest effect on the production of

infectious enveloped virus. B5 is a 42-kDa type I integral membrane glycoprotein (7, 17). Several studies have reported that removal or replacement of parts of the predicted luminal or cytoplasmic domain of B5 has little effect on incorporation of B5 into progeny virions (11, 18, 21, 23).

The A34 protein is a 24- to 28-kDa type II integral membrane glycoprotein and is involved in CEV retention on the cell surface (6, 24, 46). Deletion of A34R causes an increase in EEV production with a reduced specific infectivity. The extracellular domain of A34 has homology with C-type lectins, and a single point mutation in this domain, found in the IHDJ strain of vaccinia virus, accounts for a 50-fold increase in EEV release (3). While phenotypic characterization of recombinant viruses that contain deletions of A34R has been carried out, a specific mechanism of defect has not been characterized. In this report, we utilize a recombinant virus that expresses B5-green fluorescent protein (B5-GFP) to analyze defects in morphogenesis when the A34R gene is deleted. Subcellular characterization reveals that B5 requires A34 for proper targeting from the endoplasmic reticulum (ER) to the site of wrapping and inclusion into progeny virions.

MATERIALS AND METHODS

Cells and viruses. HeLa cell monolayers were grown in Dulbecco's modified Eagle's medium. BS-C-1 and RK13 cell monolayers were grown in Earl's minimum essential medium supplemented with 10% fetal bovine serum. All viruses were derived from the WR strain of vaccinia virus. Recombinant viruses vTF7-3 (9), vB5R-GFP (43), and v41 (46) (hereafter called vΔA34R), along with plasmid pB5R-GFP (43) and a plasmid having B5R-GFP under the control of the T7 promoter in pCDM8 (41), were a gift from Bernard Moss and have been described previously. Vaccinia virus open reading frames are designated by a capital letter indicating a HindIII restriction endonuclease fragment, a number indicating the position in the HindIII fragment, and a letter (L or R) indicating the direction of transcription, e.g., A34R. The corresponding protein is designated by a capital letter and number, e.g., A34. To construct vB5R-GFP/ΔA34R, HeLa cells were infected with vΔA34R and transfected with pB5R-GFP. Recombinant viruses that had their normal copy of B5R replaced with B5R-GFP

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were isolated and amplified as described previously. Plaque assays and time courses looking at B5R-GFP expression were carried out as previously described (43).

Plasmid constructs. The expression plasmid pBMW-118, which encodes the red fluorescent protein HcRed under the control of the modified H5 vaccinia virus promoter, has been described previously (40). To construct p118-A34R, A34R plus 500 bp of flanking sequence was amplified by PCR and inserted into pBMW-118 by ligation. Plasmids p118-A34R^{Δ2-18} and p118-A34R^{Δ40-168} were constructed by two-step PCR, using overlapping primers that produced a PCR product that had the relevant sequences removed and 500 bp of flanking sequence that was ligated into pBMW-118. To add a V5 epitope tag to the above constructs, the sequences were amplified by PCR, using an upstream primer that added the coding sequence for the tag directly after the start codon of A34R, and the products were inserted into pcDNA3 (Invitrogen) by ligation. All constructs were verified by sequencing.

Immunoprecipitation. For coimmunoprecipitation, HeLa cells were infected with vTF7-3 at 5 PFU per cell and transfected 2 h later with the appropriate plasmid, using Lipofectamine (Invitrogen) according to the manufacturer's instructions. Cytosine arabinoside (AraC) was present throughout the infection at a concentration of 40 µg per ml. The following day, cells were harvested by being scraped, washed once in phosphate-buffered saline (PBS), resuspended in lysis buffer (half-strength PBS, 1% NP-40, and phenylmethylsulfonyl fluoride), incubated on ice for 20 min, and clarified by centrifugation for 30 min at 20,000 × g. Clarified lysates were pretreated with protein G-agarose (CalBiochem) for 2 h, followed by low-speed centrifugation to remove bound proteins. Treated lysates were incubated with either an anti-V5 monoclonal antibody (MAb; Invitrogen) or MAb 19C2 (anti-B5) (34), which recognizes the luminal domain of B5, followed by protein G-agarose. Bound complexes were washed three times in lysis buffer, resuspended in protein gel loading buffer, boiled, resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to nitrocellulose membranes. Membranes were incubated with either the anti-V5 or anti-B5 MAb, followed by horseradish peroxidase-conjugated anti-mouse or anti-rat antibody, respectively (Jackson ImmunoResearch Laboratories). Bound antibodies were detected with chemiluminescence reagents (Pierce) as directed by the manufacturer. To check for expression, clarified lysates were mixed with protein loading buffer, resolved by SDS-PAGE, and analyzed by Western blotting as described above.

For radiolabeled immunoprecipitation, RK₁₃ cells were infected at 10 PFU per cell with either vB5R-GFP or vB5R-GFP/ΔA34R. At 2 h postinfection (p.i.), the inoculum was removed and replaced with medium containing [³⁵S]methionine and [³⁵S]cysteine (Perkin-Elmer). At 24 h p.i., the medium was removed from the cells, centrifuged at 360 × g to clarify, and then centrifuged through a 36% sucrose cushion at 87,500 × g for 20 min to pellet virions. Viral pellets were resuspended in 0.5 ml of lysis buffer and equilibrated using scintillation counting. B5-GFP was immunoprecipitated with anti-B5 MAb as described above. The resulting immune complexes were resuspended in protein gel loading buffer, resolved by SDS-PAGE, transferred to nitrocellulose, and visualized by autoradiography.

Virus purification. CsCl purification was carried out as described previously (42). Briefly, monolayers of RK₁₃ cells were infected at 10 PFU per cell as described above. At 18 h p.i., the medium was removed from the infected cells. Detached cells and larger debris were removed by low-speed centrifugation. Virions in the medium were centrifuged through a 36% sucrose cushion, resuspended in swelling buffer with sonication, and banded on a preformed CsCl step gradient as previously described. Gradients were fractionated from the bottom of the tube, and the amount of radiation present in each fraction was determined by scintillation counting.

Subcellular fractionation. HeLa cells grown in T-175 flasks were infected with either vB5R-GFP or vB5R-GFP/ΔA34R at a multiplicity of infection (MOI) of 5.0. After 2 h of infection, the inoculum was replaced with fresh medium. At 12 h p.i., the cells were harvested by being scraped into medium, collected by low-speed centrifugation, resuspended in 1 ml of homogenization buffer (0.25 M sucrose, 1 mM EDTA, 10 mM HEPES, pH 7.4) containing 0.2 mM phenylmethylsulfonyl fluoride and protease inhibitor cocktail tablets (Roche Diagnostics), and broken by passage through a 27G syringe needle 10 times. The nuclei and unbroken cells were removed by centrifugation at 2,400 × g for 5 min. The postnuclear supernatant was loaded onto a 10.5-ml preformed 5 to 25% continuous iodixanol gradient (Axis Shield) and centrifuged at 200,000 × g for 2.5 h at 4°C. After centrifugation, 0.8-ml fractions were collected from the bottom of the tube. The protein in each fraction was precipitated with trichloroacetic acid and washed twice with acetone. Precipitated proteins were resuspended in LDS sample buffer (Invitrogen), resolved in a 4 to 12% bis-Tris gel (Invitrogen), and transferred to nitrocellulose membranes. Nitrocellulose membranes were incu-

bated with rabbit anti-calnexin antibody (Stressgen), followed by horseradish peroxidase-conjugated donkey anti-rabbit (Jackson ImmunoResearch Laboratories) and anti-golgin-97 (Invitrogen, Molecular Probes) MAbs, followed by horseradish peroxidase-conjugated donkey anti-mouse (Jackson ImmunoResearch Laboratories), to determine the fractions containing ER and Golgi components, respectively. The B5-GFP proteins were detected by an anti-GFP MAb (Covance). Bound antibodies were detected with a chemiluminescence substrate (Pierce) following the manufacturer's instructions.

Fluorescence microscopy. HeLa cells were grown to confluence on coverslips and infected with 1 PFU per cell. For complementation studies, cells were transfected at 2 h p.i. as described above, in the absence of AraC. The following day, infected cells were fixed with 4% paraformaldehyde and permeabilized with Triton X-100, both in PBS. To stain total B5 protein in the cell, fixed and permeabilized cells were incubated with anti-B5 MAb followed by Texas Red-conjugated goat anti-rat antibody (Jackson ImmunoResearch Laboratories). Stained cells on coverslips were mounted in Mowiol containing 1 µg of 4',6'-diamidino-2-phenylindole dihydrochloride (DAPI; EM Sciences) per ml to visualize DNA in the nucleus and viral factories. To stain B5 on the cell surface, fixed cells were quenched with 0.1 M glycine, rinsed in PBS, and incubated with anti-B5 MAb followed by Texas Red-conjugated goat anti-rat antibody (Jackson ImmunoResearch Laboratories). Coverslips were stained with 50 µg of Hoechst per ml to visualize DNA and then mounted in Mowiol. For complementation studies, cells were fixed as described above and mounted directly in Mowiol. Cells were imaged using a Leica DMIRB inverted fluorescence microscope with a cooled charge-coupled device (Cooke) that was controlled using Image-Pro Plus software (Media Cybernetics).

RESULTS

Construction and characterization of a recombinant vaccinia virus that has the A34R gene deleted and the B5R gene replaced with B5R-GFP. Previous work demonstrated that the chimeric protein B5-GFP could functionally replace B5 during infection (43). In addition, a recombinant virus that has B5R replaced with the coding sequence for B5R-GFP resembles the parental virus WR and has proven to be a useful tool for the study of IEV egress in living cells (14, 30, 42–44). We thought it would be equally useful for studying viral morphogenesis in the absence of A34 and therefore replaced the normal B5R gene in vΔA34R with B5R-GFP, using a similar strategy to that described previously (43). The new recombinant (vB5R-GFP/ΔA34R) produced plaques on monolayers of BS-C-1 cells that resembled those formed by the parental virus vΔA34R and were markedly smaller than those formed by vB5R-GFP (Fig. 1).

To investigate the effect that deletion of A34R has on morphogenesis at the subcellular level, cells infected with vB5R-GFP/ΔA34R were visualized using fluorescence microscopy and compared to cells infected with vB5R-GFP. As reported previously (43), cells infected with vB5R-GFP displayed three distinct fluorescent hallmarks, at the site of wrapping in the juxtanuclear region, at the vertices of the cell, and on virion-sized particles. All three of these were absent in cells infected with vB5R-GFP/ΔA34R, and only a lacy fluorescent pattern was seen throughout the cytoplasm (Fig. 2), indicating that during infection, B5 appears to be mistargeted in the absence of A34. Western blot analysis demonstrated that the B5-GFP chimera is intact in the absence of A34 (not shown).

To ensure that the mistargeting of B5R-GFP was not the result of mutations acquired in other genes during the creation and amplification of the recombinant virus, the A34R gene and promoter were provided in *trans* on a plasmid (p118-A34R) that also contained a red fluorescent protein under control of the vaccinia virus modified H5 promoter as a marker for cells

FIG. 1. Plaque phenotypes. The indicated viruses were plated on monolayers of BS-C-1 cells. After 2 days, plaques were imaged using phase-contrast (PC) and fluorescence microscopy (GFP). After being imaged, cells were stained with crystal violet (CV).

that were both infected and transfected. Cells infected with vB5R-GFP/ΔA34R and transfected with p118-A34R fluoresced red and had a GFP fluorescence pattern identical to that of cells infected with vB5R-GFP (Fig. 2). In contrast, cells infected with vB5R-GFP/ΔA34R and transfected with the control plasmid p118 fluoresced red, indicating that they were transfected, but showed the same mistargeted GFP fluorescence pattern as that observed in cells infected with vB5R-GFP/ΔA34R.

In the absence of A34, B5-GFP is not transported to the cell surface. To further characterize the localization of B5-GFP in the absence of A34, cells infected with vB5R-GFP/ΔA34R were stained with the anti-B5 MAb. Cells permeabilized before staining showed a nearly perfect overlap of anti-B5 MAb staining and GFP fluorescence, demonstrating that GFP fluorescence is a true indicator of B5 localization (Fig. 3). Cells infected with vaccinia virus normally deposit B5 on the cell surface. To determine if our recombinant virus deposited B5-GFP on the cell surface, infected cells were stained without permeabilization with anti-B5 MAb. Cells infected with vB5R-GFP showed B5-GFP on the cell surface when they were stained without permeabilization, and importantly, the GFP signal in the juxtanuclear region was not stained in these cells, verifying that they were not permeabilized. In contrast, cells infected with vB5R-GFP/ΔA34R showed no B5-GFP surface staining (Fig. 3), indicating that in the absence of A34, B5-GFP does not traffic to the cell surface.

FIG. 2. Localization of B5-GFP in the presence and absence of A34. HeLa cells were infected with the indicated viruses and either left untransfected or transfected with the indicated plasmids. The next day, B5-GFP fluorescence was imaged in cells that were both infected and transfected (where applicable), using fluorescence microscopy. Arrowheads depict the site of wrapping, concave arrowheads depict the collection of fluorescence in the vertices of the cell, and arrows point to virion-sized particles. The insets show red fluorescence.

B5-GFP localizes predominantly to the ER in the absence of A34. The B5-GFP fluorescence pattern seen in the absence of A34 was reminiscent of ER staining. To test this hypothesis, cells infected with vB5R-GFP/ΔA34R were stained with an antibody against the resident ER marker protein di-isomerase (PDI). Cells infected with vB5R-GFP showed only coincidental overlap with PDI, in a manner that was not structurally similar between the two fluorophores (Fig. 4). In contrast, there was nearly perfect colocalization between PDI and B5-GFP in cells infected with vB5R-GFP/ΔA34R. At a higher magnification (Fig. 4, insets), similar structures could be seen to be labeled by both fluorophores, indicating colocalization.

To further examine the subcellular localization of B5-GFP, membranes from infected cells were separated in a 5 to 25% continuous iodixanol gradient. Fractions from the gradients were analyzed by Western blotting with antibodies that recognize resident ER and *trans*-Golgi network proteins. In cells infected with vB5R-GFP, B5-GFP was found predominantly in lighter fractions that were not associated with the ER marker calnexin (Fig. 5). In contrast, the majority of B5-GFP in cells infected with vB5R-GFP/ΔA34R was found in heavier fractions with the ER marker calnexin (Fig. 5). Taken together, all of our data indicate that in the absence of A34, the majority of B5-GFP is not transported out of the ER to the site of wrapping.

FIG. 3. Localization of B5-GFP in infected cells. HeLa cells were infected with the indicated recombinant viruses and stained, either with or without permeabilization, with anti-B5 MAb followed by Texas Red-conjugated goat anti-rat antibody (red). Green fluorescence represents B5-GFP, and blue is either DAPI (permeabilized) or Hoechst (unpermeabilized) staining of DNA. Overlapping red and green signals are shown in yellow.

The luminal domain of A34 is required for proper targeting of B5-GFP. B5 and A34 are type I and type II transmembrane proteins, respectively. Both have single luminal, transmembrane, and cytoplasmic domains. To determine which domain of A34 is responsible for the proper targeting of B5 during infection, we tested two constructs for complementation of localization *in trans*. The first construct (p118-A34R Δ^{2-18}) had residues 2 to 18 of A34 removed, which represents most of the predicted cytoplasmic domain. The second construct (p118-A34R Δ^{40-168}) had residues 40 to 168 of A34 removed, which represents most of the predicted luminal domain. Cells infected with vB5R-GFP/ Δ A34R and transfected with p118-A34R Δ^{40-168} displayed a GFP fluorescence pattern like that seen in cells infected with vB5R-GFP/ Δ A34R alone (Fig. 6), indicating that neither the cytoplasmic nor the transmembrane

domain is sufficient to retarget B5-GFP to the site of wrapping. In contrast, cells infected with vB5R-GFP/ Δ A34R and transfected with p118-A34R Δ^{2-18} showed GFP fluorescence accumulation in the juxtanuclear region, indicating that the luminal domain of A34 is required for proper targeting of B5 to the site of wrapping during infection (Fig. 6).

The luminal domain of A34 interacts with B5-GFP. The most straightforward explanation for our results is that A34 and B5 interact through their luminal domains and that this interaction is required for the proper targeting of B5-GFP during infection. Indeed, an interaction between B5 and A34 has been reported (33). To look for an interaction between A34 and B5-GFP, we added the coding sequence for the V5 epitope tag to the 5' terminus of the A34R gene under control of the T7 promoter. The resulting plasmid (pV5-A34R) was

FIG. 4. Localization of B5R-GFP and PDI in infected cells. HeLa cells were infected with the indicated recombinant viruses and stained with anti-PDI MAb followed by Texas Red-conjugated goat anti-mouse antibody (red), followed by DAPI to label DNA (blue). Green fluorescence represents B5-GFP. Boxed regions are enlarged to show structural details. Overlapping red and green signals are shown in yellow.

transfected along with pT7-B5R-GFP, which has B5R-GFP under control of the T7 promoter, into cells that were infected with a vaccinia virus that expresses the T7 polymerase (vTF7-3). Many of the EV-specific proteins, including A33 (32), A34 (6), A36 (26), A56 (4), B5 (43), and F13 (12), have been shown to be produced predominantly late in infection, after replication of the viral genome. To block late expression and reduce

the amount of endogenous A34 and B5 which is untagged and would compete for interaction in our assay, late viral expression was blocked by the addition of AraC (5). The lysate from transfected/infected cells was incubated with anti-B5 MAb followed by protein G-agarose. Bound proteins were separated by SDS-PAGE, blotted onto nitrocellulose, and probed with an anti-V5 MAb. V5-A34 was precipitated with the anti-B5 MAb

FIG. 5. Subcellular fractionation. HeLa cells were infected with either vB5R-GFP or vB5R-GFP/ΔA34R at an MOI of 5.0. At 12 h p.i., cells were harvested, and the postnuclear supernatant was loaded onto a 5 to 25% continuous iodixanol gradient, centrifuged, and fractionated. Trichloroacetic acid-precipitated proteins were analyzed by SDS-PAGE, followed by Western blotting with anti-calnexin antibody, anti-golgin-97 MAb, and anti-GFP MAb.

FIG. 6. *trans*-Complementation. HeLa cells were infected with vB5R-GFP/ΔA34R and transfected with the indicated plasmids. The next day, B5-GFP fluorescence was imaged in cells that were both infected and transfected, using fluorescence microscopy. Arrowheads point to the site of wrapping, concave arrowheads depict the collection of fluorescence in the vertices of the cell, and arrows point to virion-sized particles. The insets show red fluorescence.

(Fig. 7A), indicating a direct interaction. Similarly, B5-GFP was precipitated from the same lysate by the anti-V5 MAb (not shown). To determine if the luminal or cytoplasmic domain of A34 interacted with B5, V5 epitope-tagged versions of our A34R truncations A34R^{Δ2-18} and A34R^{Δ40-168} (pV5-A34R^{Δ2-18} and pV5-A34R^{Δ40-168}, respectively) were coexpressed with B5-GFP in cells, using the vaccinia virus T7 expression system, and interactions were detected using

coimmunoprecipitation with the anti-V5 MAb. Although both A34 constructs were expressed (Fig. 7C), B5-GFP was coimmunoprecipitated with the V5 MAb only when it was coexpressed with V5-A34^{Δ2-18}, not with V5-A34^{Δ40-168} (Fig. 7B), indicating that B5-GFP interacts with the luminal domain of A34.

B5 incorporation into EEV. It was previously reported that a recombinant virus that has A34R deleted still produces wrapped virus and releases ~25-fold more EEV than the parental WR strain (24). In cells infected with vB5R-GFP/ΔA34R, B5-GFP was not detected on the cell surface and GFP-labeled virion-sized particles were not observed, indicating that our recombinant may be defective for producing wrapped virions or that the virions produced do not contain B5. To investigate these possibilities, cells infected with either vB5R-GFP or vB5R-GFP/ΔA34R were incubated in medium containing [³⁵S]methionine and [³⁵S]cysteine to radiolabel the proteins incorporated into progeny virions. Virions released into the medium, which were predominately EEV, were purified by CsCl density gradient centrifugation and fractionation. Both recombinants released virions into the medium, resulting in peaks in the gradient which were representative of EEV (Fig. 8A). As reported previously, the deletion of A34 resulted in increased amounts of EEV (Fig. 8A) (24). To determine if the EEV produced by cells infected with vB5R-GFP/ΔA34R contained B5-GFP, RK₁₃ cells were infected as described above, and virions released into the medium were centrifuged through a 36% sucrose cushion. The resulting virion pellets were disrupted using detergent, and the lysates were equilibrated to each other using scintillation counting. Equilibrated

FIG. 7. Coimmunoprecipitation. HeLa cells were infected with vTF7-3 at an MOI of 5 and transfected with the indicated plasmids. The next day, cells were lysed, and the lysates were immunoprecipitated with either anti-B5 MAb (A) or anti-V5 MAb (B). Immune complexes were analyzed by SDS-PAGE followed by Western blotting with either anti-V5 MAb (A) or anti-B5 MAb (B). (C) Cell lysates from panel B were checked for expression of truncated A34 by Western blotting with anti-V5 MAb, with β-tubulin as a loading control. The masses (in kilodaltons) and positions of marker proteins are shown to the left of each blot. The positions of cross-reacting heavy and light chains (HC and LC, respectively) from the precipitating B5 MAb, along with the precipitated proteins, are labeled on the right of each blot.

FIG. 8. EEV production. RK₁₃ cells were infected with 10 PFU of the indicated viruses. After 2 h, cells were labeled with 500 μ Ci of [³⁵S]methionine and [³⁵S]cysteine. Particles in the medium were concentrated by sedimentation through a sucrose cushion. (A) Sedimented virions were applied to CsCl density gradients and centrifuged. The amounts of radioactive material in the fractions were determined by scintillation counting. (B) Sedimented virions were lysed with detergent and equilibrated by scintillation counting. Equal volumes of the equilibrated lysates were precipitated with anti-B5R MAb. Immune complexes (left) and equal volumes of equilibrated lysates (right) were analyzed by SDS-PAGE followed by autoradiography and densitometry. The relative amounts of signal are shown at the bottom. The masses (kilodaltons) and positions of marker proteins are shown between the blots.

lysates were incubated with the anti-B5 MAb followed by protein G-agarose. Bound proteins were separated by SDS-PAGE and blotted onto nitrocellulose, and radiolabeled bands were detected by exposure to film. A band of the predicted size for B5-GFP was clearly seen for virions produced by cells infected with vB5R-GFP (Fig. 8B). In contrast, there was not a corresponding band in lysates from virions produced by vB5R-GFP/ Δ A34R. Lysates were examined to be sure that there were approximately equal amounts of virions in the equilibrated lysates (Fig. 8B). Although there were more virions released by cells infected with vB5R-GFP/ Δ A34R, densitometric analysis revealed that they contained $\sim$ 20-fold less B5-GFP than did virions released by cells infected with vB5R-GFP.

DISCUSSION

With the exception of F13, none of the IEV proteins are predicted to have enzymatic functions, and therefore they

probably act as scaffolding for other viral or cellular proteins that mediate intracellular wrapping. Interactions between A33 and A36 (33, 47), A34 and B5, A34 and A36 (33), A33 and B5 (29), and B5 and F13 (27) have been described. With the exception of the A33-A36 interaction (44, 47), there is little known about the functions that these interactions have during morphogenesis. A33 is thought to require B5 for incorporation into EV (29). Our data suggest that B5 requires A34 for incorporation into EV and therefore that A33 should require A34, through B5, for incorporation into EV. Since interactions between A33 and A34 have also been reported, it will be of interest to see if these three proteins form a trimeric complex or associate independently.

B5R and A34R are highly conserved among orthopoxviruses, and their gene products are thought to be involved in both IEV formation and cell-to-cell spread. An interaction between B5 and A34 was previously reported (33). Our work confirms this initial report and extends it to show that the region of interaction is in the luminal domains of both proteins and that the interaction is required for the proper targeting of B5 during infection and for subsequent incorporation into EEV. A previous study of a recombinant virus that had A34R deleted reported that the incorporation of other known EEV-specific proteins into EEV did not require A34 (24). Upon reevaluation of those data, a significant reduction in the amount of detected B5 in the purified EEV was evident, supporting the findings presented here.

Why does B5 need A34 for proper targeting during infection? We can think of two possibilities for why B5 does not exit the ER in the absence of A34. First, A34 acts as a chaperone for the proper folding of B5 and its export from the ER. Alternatively, the binding of A34 to B5 prevents B5 from interacting with a third protein in the ER that prevents its export. Several reports have shown that B5 expressed in uninfected cells appears to traffic normally to the juxtanuclear region (18, 22, 41), indicating that it is competent to fold and be exported in the absence of other viral proteins, including A34. These reports also imply that if B5 interacts with a second protein that prevents its export from the ER, then this second protein is most likely encoded by the virus.

Other viral glycoproteins have been shown to be dependent on a second protein for their proper subcellular localization during infection. The gH glycoprotein from herpesviruses requires gL for proper folding and subsequent localization to progeny virions and the cell surface (16). Similarly, the Gc protein from bunyaviruses requires interaction with Gn for proper trafficking to the Golgi apparatus (19, 35). It is noteworthy that, like orthopoxviruses, both of these viruses acquire their envelope from an intracellular organelle. It is unclear at this time if there is a functional significance between these glycoprotein interactions, subcellular trafficking, and intracellular envelopment.

The double membrane surrounding the EEV presents a topological problem of how the viral core escapes two membranes to enter the cytoplasm. A recent article reported that the addition of glycosaminoglycans (GAGs) to EEV caused the nonfusogenic dissolution of the outer EEV membrane and the release of single-membrane-bound IMV (20), which can then penetrate the cell and release the core into the cytoplasm by using more traditional viral entry mechanisms.

GAG-dependent dissolution did not occur in EEV formed by recombinant viruses that had either A34R or B5R deleted, indicating that B5 and A34 may be involved in binding of GAGs and/or the EEV membrane dissolution pathway. In accordance with this notion, it was reported that freeze-thawing EEV that were formed in the absence of A34 increased their infectivity by releasing the IMV contained within, suggesting that the EEV membrane is more stable in the absence of A34 (24). This was later verified by microscopy, confirming the stability of the EEV membrane in the absence of A34 (15). Our results show that the amount of B5 in EEV formed in the absence of A34 is greatly reduced and may explain why v Δ A34R and v Δ B5R are similarly defective in EEV membrane dissolution. Furthermore, our results suggest that A34 may not be involved directly in the dissolution process and that B5 may be the main glycoprotein involved in EEV attachment to cells and activation of the dissolution pathway. Taken together, these results concur with previous data showing that cells infected with v Δ A34R release many more virions than do WR-infected cells but that the particles have a much lower specific infectivity and require physical removal of their outer membrane to be infectious (24).

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