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Vaccinia Virus A26 and A27 Proteins Form a Stable Complex Tethered to Mature Virions by Association with the A17 Transmembrane Protein[▽]

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During vaccinia virus replication, mature virions (MVs) are wrapped with cellular membranes, transported to the periphery, and exported as extracellular virions (EVs) that mediate spread. The A26 protein is unusual in that it is present in MVs but not EVs. This distribution led to a proposal that A26 negatively regulates wrapping. A26 also has roles in the attachment of MVs to the cell surface and incorporation of MVs into proteinaceous A-type inclusions in some orthopoxvirus species. However, A26 lacks a transmembrane domain, and nothing is known regarding how it associates with the MV, regulates incorporation of the MV into inclusions, and possibly prevents EV formation. Here, we provide evidence that A26 forms a disulfide-bonded complex with A27 that is anchored to the MV through a noncovalent interaction with the A17 transmembrane protein. In the absence of A27, A26 was unstable, and only small amounts were detected. The interaction of A26 with A27 depended on a C-terminal segment of A26 with 45% amino acid identity to A27. Deletion of A26 failed to enhance EV formation by vaccinia virus, as had been predicted. Nevertheless, the interaction of A26 and A27 may have functional significance, since each is thought to mediate binding to cells through interaction with laminin and heparan sulfate, respectively. We also found that A26 formed a noncovalent complex with A25, a truncated form of the cowpox virus A-type inclusion matrix protein. The latter association suggests a mechanism for incorporation of virions into A-type inclusions in other orthopoxvirus strains.

Vaccinia virus (VACV), a member of the family *Poxviridae*, undergoes an elaborate assembly process involving the sequential formation of morphologically distinct infectious virus particles (6). The mature virions (MVs) are the most abundant infectious particles and are released upon cell lysis. The MV consists of (i) a dumbbell-shaped core containing the viral double-stranded DNA genome, structural proteins, and enzymes and factors for early gene transcription; (ii) lateral bodies of undefined nature that are nestled in the concavities of the core; and (iii) an external lipoprotein membrane. Membranes derived from either the *trans*-Golgi network or endosomal cisternae envelop a subset of MV particles to form doubly wrapped virions (WVs) (11, 27, 31). WVs traffic along microtubules to the periphery of the cell, where the outer membrane fuses with the plasma membrane to release enveloped virions (EVs) (12, 23, 36, 37). Many EVs remain associated with the outer surface of the plasma membrane and mediate spread to neighboring cells on the tips of actin-containing microvilli (2, 30). Some poxviruses (e.g., cowpox, ectromelia, raccoonpox, fowlpox, and canarypox viruses, but not VACV or variola virus) occlude MVs in a proteinaceous body called an A-type inclusion (ATI) that is thought to protect the virus particles under harsh environmental conditions (17).

Recent mass spectroscopy studies indicate that MVs are comprised of approximately 80 polypeptides (5, 22, 39). Although EVs are essentially MVs with an outer membrane, there are some differences. Notably, VACV proteins A25 (VACVWR148) and A26 (VACVWR149) are present in MVs but absent from EVs (33). A25 has a predicted mass of 84 kDa and is a truncated, apparently nonfunctional homolog of the cowpox ATI matrix protein (1, 20, 21). VACV A26 is a 58-kDa full-length homolog of the occlusion factor required for incorporating MVs into the ATI matrix (19). Nevertheless, A26 is conserved in orthopoxviruses irrespective of their capacity to form ATIs, suggesting an additional role. Ulaeto and coworkers (33) suggested that A26 acts as a switch to enhance the production of MVs at the expense of EVs. Following up on this idea, McKelvey et al. (19) suggested that A26 might enhance retrograde transport of MVs. However, the effect of deleting the A26 gene on EV production has not been reported. More recently, A26 was shown to bind laminin and to function in cell attachment (3).

In the present study, we showed that A26 exists in a complex with three other viral proteins: A25, A27, and A17. The interaction of A26 and A27 was direct and stabilized by a disulfide bond. The interaction of A26 with the A17 transmembrane protein was mediated through A27, and this provided the anchor for the localization of A26 on the surfaces of MVs. The interaction of A26 with A27 is of interest because both proteins have roles in virus attachment to cells (3). Since A27 is required for EV formation (24), the interaction of A26 and A27 also provided a plausible mechanism for A26 to negatively regulate wrapping. However, EV formation was not enhanced by deletion of A26. The interaction of A26 with A25 has

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special significance because it may provide a mechanism for the incorporation of MVs in ATIs (19).

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MATERIALS AND METHODS

Cells and viruses. African green monkey kidney BS-C-1, rabbit kidney epithelial RK-13, and human HeLa S3 cell cultures were maintained in minimum essential medium with Earle's salts (Quality Biological, Gaithersburg, MD) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 units/ml of penicillin, and 100 μg/ml of streptomycin. Infections were carried out in medium containing 2.5% fetal bovine serum and otherwise supplemented as described above. Recombinant viruses derived from the VACV Western reserve (WR) strain were propagated as described previously (8).

Antibodies for Western blotting. Rabbit polyclonal antibodies against the following VACV proteins were used: A27 (10), A17 (38), and A3 (R. Doms and B. Moss, unpublished data). Anti- hemagglutinin (HA) (Bethyl Laboratories, Inc., Montgomery, TX) and anti-V5 (Invitrogen, Carlsbad, CA) antibodies were conjugated to horseradish peroxidase.

Recombinant virus and plasmid construction. The A27 deletion (vYFP-A4/ΔA27, referred to here as vΔA27) and revertant (vYFP-A4/ΔA27-rev, referred to here as vΔA27.Rev) viruses and vA4-YFP, used to generate vΔA27, were previously described (28, 35). Both express the yellow fluorescent protein fused to the N terminus of A4 and have A27L deleted from its native locus. vΔA27.Rev has a full-length copy of the A27L gene under its native promoter in the thymidine kinase locus (35). The A26 deletion virus (vΔA26) was generated from a recombinant VACV WR virus that expresses luciferase via a synthetic early-late promoter (WRvFire) (32) by replacing the A26L gene with a cassette that expresses the enhanced green fluorescent protein. vA26V5 was derived from VACV WR by homologous recombination with a PCR product containing the A26L gene with a C-terminal V5 epitope tag (GKPIPNPLLGLDST) under the control of the A26L promoter and the enhanced green fluorescent protein open reading frame (ORF) regulated by the late promoter derived from the A17R gene, and A26L downstream flanking sequences. Recombinant viruses expressing enhanced green fluorescent protein were clonally purified by four consecutive rounds of plaque isolation. Genome modifications were verified by sequencing.

pA26V5 was generated by PCR amplification of V5-tagged A26L from vA26V5 genomic DNA using the forward primer 5′-CTTAACTCTTTTGTTAATTAAAAGTATATTCAAAAAATGAG-3′ and the reverse primer 5′-CTACGTAGAATCGAGACCGAGGAGAGGGTTAGGGATAGGCTTACC-3′. The resulting PCR product included the A26L ORF and a 48-bp upstream region containing the predicted late promoter consisting of an A/T-rich region and the TAAATG initiator element at the −1 position (7). pA26ΔCV5 contains the A26L sequence coding for amino acids 1 to 409 of A26 plus a C-terminally appended V5 tag under the control of the A26L promoter sequence as described above. Primers 5′-CTTAACTCTTTTGTTAATTAAAAGTATATTCAAAAAATGAG-3′ and 5′-CTACGTAGAATCGAGACCGAGGAGAGGGTTAGGGATAGGCTTACCTTCTTCTACAGGAAGAAGTTTCGGCCTC-3′ (the V5 sequence is underlined) were used to amplify the A26 sequence coding for the N-terminal 409 amino acids from VACV WR DNA. pA26ΔNV5 expresses the C-terminal 220 amino acids of A26 fused to a C-terminal V5 epitope tag. The forward primer 5′-GAAAAATTTAGCAATGATGCTATACTCGTTTATATTAGAACAAAC-3′ and the reverse primer 5′-CTACGTAGAATCGAGACCGAGGAGAGGGTTAGGGATAGGCTTACC-3′ were used to amplify the C-terminal fragment of A26 from vA26V5 DNA. To add the A26 promoter sequence, the above-mentioned PCR product was used as a template in a reaction using the primers 5′-CTTAACTCTTTTGTTAATTAAAAGTATATTCAAAAAATGAGTTATATAAATGGAAAAATTTAGCAATGATGCTATACTCG-3′ (the A26 upstream sequence including the promoter is underlined) and 5′-CTACGTAGAATCGAGACCGAGGAGAGGGTTAGGGATAGGCTTACC-3′. A plasmid including the A27L ORF and the upstream 49 bp shown to be sufficient to drive expression of A27 (35) was also generated. All recombinant PCR products were amplified using Accuprime *Pfx* (Invitrogen), blunt-end ligated into pCR-BluntII-TOPO (Invitrogen), and verified by DNA sequencing.

The PCR product used to construct pT7.A26V5 was generated using primers 5′-GGCCG**C/TCGAG**ATGGCGAACATTATAAATTTATGGAACGGAATTGTACC-3 and 5′-GG**CG/TCGAC**CTACGTAGAATCGAGACCGAGGAGAGG-3′, which introduced N-terminal XhoI and C-terminal SalI restriction sites (boldface), respectively, for directional cloning into the pTNT vector (Promega, Madison, WI) under the control of the T7 promoter. Similarly, the A27L gene was amplified with primers 5′-GCCCGG**C/TCGAG**ATGGACGGAACTCTTTTCCCCGGAGATG-3′ and 5′-GGC**G/TCGAC**CTAAGCGTAGTCTGGGACGTCGTATGGGTACTCATATGGGCGCCGTCCAGTCTG-3′, thereby introducing the influenza virus HA epitope tag (YPYDVPDYA) before the stop codon to generate pT7.A27HA.

Transient expression of proteins. Six-well plates of BS-C-1 cells were infected at a multiplicity of infection of 5 PFU per cell in Opti-MEM reduced-serum medium (Invitrogen) for 1 h. Following adsorption, the monolayers were washed twice with Opti-MEM, and 1 μg of each plasmid in 8 μl of Lipofectamine 2000 (Invitrogen) in fresh Opti-MEM (Invitrogen) was added. The monolayers were harvested after 18 to 20 h.

Virus purification. MVs were purified from HeLa cell extracts by sedimentation through two 36% (wt/vol) sucrose cushions and banding once on a 25 to 40% (wt/vol) sucrose gradient as described previously (9). For CsCl gradient purification, approximately 6×10^7 BS-C-1 cells in two T150 flasks were infected with vΔA27 at a multiplicity of infection of 5 PFU per cell and then transfected with pA26V5 in the presence or absence of pA27 (1 μg of each plasmid per 1×10^6 cells). After 24 h, the cells were harvested and subjected to two 36% sucrose cushions followed by banding on a 25 to 40% sucrose gradient as described above. The sucrose gradient-purified virus was resuspended in 500 μl of 10 mM Tris-HCl (pH 9.0), layered onto a preformed CsCl gradient (1.23 g/ml to 1.29 g/ml), and centrifuged at 180,000 × g for 4 h at room temperature as described previously (23).

Immunoaffinity purification and Western blot analysis. Intact cells or sucrose gradient-purified virions were solubilized in lysis buffer (150 mM NaCl, 50 mM Tris-HCl, pH 8.0, 1% NP-40) for 30 min on ice. Following brief sonication, the extracts were clarified by centrifugation and incubated with unconjugated agarose A beads (Invitrogen) for 1 h at 4°C. The extracts were then rotated for 4 to 12 h at 4°C with antiserum prior to incubation with prewashed agarose A beads overnight at 4°C. Anti-V5 immunoaffinity purification was carried out according to the manufacturer's instructions using the above-mentioned lysis buffer. Bound fractions were eluted in lithium dodecyl sulfate (LDS) loading buffer (Invitrogen), resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 4 to 12% NuPAGE Bis-Tris gels in NuPAGE MOPS (morpholinepropanesulfonic acid) running buffer (Invitrogen), transferred to a nitrocellulose membrane, and analyzed by Western blotting using rabbit polyclonal antiserum and anti-rabbit immunoglobulin G conjugated to horseradish peroxidase (Pierce, Rockford, IL) consecutively or with anti-V5 antibody and anti-HA–horseradish peroxidase antibodies as described above. Bound immunoglobulin G was detected using the SuperSignal chemiluminescent substrates (Pierce). For analysis of whole-cell lysates, cells were collected by centrifugation and resuspended in LDS sample buffer (Invitrogen) prior to SDS-PAGE. *N*-Ethylmaleimide (NEM) (Sigma) was added to the cells prior to lysis as previously described (29).

Biotinylation of purified virions. Sucrose gradient-purified virions were rotated with 1 mg/ml of sulfosuccinimidyl 2-(biotinamido)-ethyl-1,3-dithiopropionate (EZ-Link sulfo-NHS-SS-biotin; Pierce) for 30 min at 4°C. Excess biotin was quenched with 50 mM Tris-HCl, pH 8.0. Virions were pelleted by centrifugation at 20,000 × *g* for 30 min at 4°C and solubilized by the addition of SDS-PAGE sample buffer. To recover the biotinylated proteins, samples were rotated with NutrAvidin agarose beads (Pierce) for 1 h at 4°C. The supernatant fraction was removed, and the beads were washed five times in cold phosphate-buffered saline (Quality Biological, Inc.). The biotinylated proteins were then eluted using LDS sample buffer plus 50 mM dithiothreitol and analyzed along with the unbound fractions by SDS-PAGE and Western blot analysis.

EV release assay. RK-13 and HeLa cells were infected with VACV strain WR or vΔA26 at a multiplicity of infection of 0.1 PFU per cell. After 48 h, the supernatant, which contained the EV, was removed and clarified using low-speed centrifugation. The monolayers, containing MV, were washed and lysed by freeze-thawing. The titers of virus in both supernatants and cell lysates were determined using BS-C-1 cells, and supernatants and cell lysates were overlaid with medium containing 2.5% fetal bovine serum plus 0.5% methylcellulose. After 48 h, the plaques were stained with crystal violet and counted. To measure adherent EVs, the cells were treated with various amounts of trypsin in medium without fetal bovine serum for 45 min at 37°C (2). The supernatants were removed from the monolayers and clarified by low-speed centrifugation prior to titration as described above.

RESULTS

Functional interaction of A26 with A27. During an analysis of proteins in MVs purified from cells infected with the A27L deletion mutant vΔA27, we noticed that a 60-kDa polypeptide

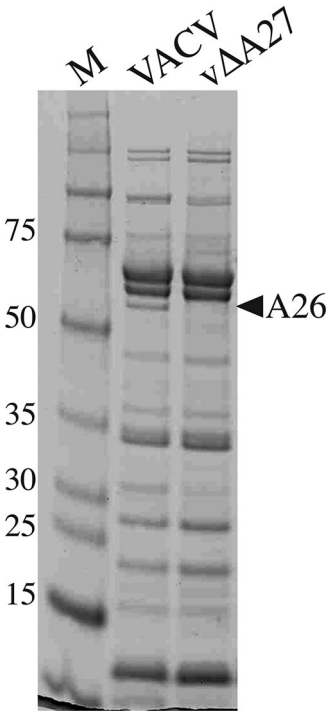

FIG. 1. Absence of A26 in virions lacking A27. Virions from cells infected with vΔA27 and the parental virus vA4-YFP (VACV) were purified by sucrose gradient sedimentation, disrupted with LDS and reducing agent, and analyzed by SDS-PAGE. Polypeptides were detected by staining them with Coomassie blue. The masses of marker proteins (M) are in kDa. The arrowhead labeled A26 points to the position of the 60-kDa band present in VACV and missing from vΔA27.

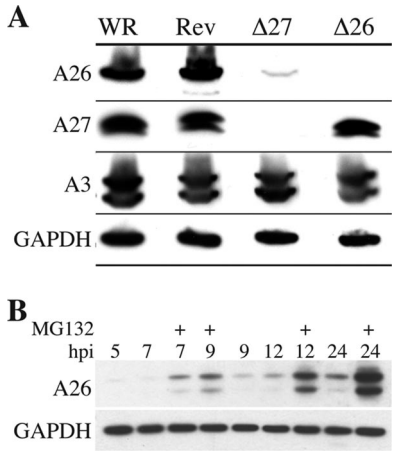

FIG. 2. Decreased cytoplasmic A26 in the absence of A27. (A) Western blot. Extracts of cells infected for 24 h with VACV WR (WR), vΔA27.Rev (Rev), vΔA27 (Δ27), or vΔA26 (Δ26) were reduced and analyzed by SDS-PAGE, and the proteins were identified by Western blotting using antibodies to A26, A27, A3, and GAPDH as indicated. (B) Effect of proteasome inhibitor. Cells were infected with vΔA27. After 5 h, replicate cultures were treated with 10 μM MG-132 (+) or left untreated, and the incubation was continued. At the indicated times, lysates were prepared and analyzed by Western blotting with antibodies to A26 and GAPDH. hpi, hours postinfection.

was missing (Fig. 1). The band from the parental virus corresponding to the missing 60-kDa protein was excised from the gel, digested with trypsin, and shown to be A26 by mass spectrometry. Our initial thought was that A27 was required only for incorporation of A26 into MVs. However, extracts of cells infected with vΔA27 had very small amounts of A26 compared to the amount from cells infected with the parental virus (Fig. 2A). The deficiency of A26 was specific, since the viral A3 protein and cellular glyceraldehyde-3-phosphate dehydrogenase (GAPDH). were present in normal amounts. There was no reciprocal effect, i.e., deletion of the ORF encoding A26 did not cause a decrease in A27 (Fig. 2A).

A trivial explanation for the deficiency of A26 would be an unintentional mutation of the A26L promoter or ORF during the deletion of the neighboring A27L gene. However, DNA sequencing indicated that no such mutation had occurred. Furthermore, a normal amount of A26 was detected by Western blotting of extracts from cells infected with the revertant virus vΔA27Rev (35), in which a new copy of A27 with its natural promoter had been inserted into the VACV thymidine kinase locus, thereby leaving unaltered the site of the original A27L deletion adjacent to A26L (Fig. 2A). Thus, the synthesis of A27 was necessary for normal amounts of A26.

The above data suggested that A27 might be required for the stability of A26. To investigate whether A26 was being actively degraded, cells were infected with vΔA27 and the specific proteasome inhibitor MG132 was added 5 h later. The levels of A26, determined by Western blotting, increased greatly over time in the presence of MG132 (Fig. 2B), indicating that A26 was being degraded by the proteasome in the absence of A27. In contrast, MG132 reduced A26 somewhat in the presence of A27, presumably due to a general effect (data not shown). We also found that the amount of A26 in cells infected with vΔA27 could be increased by the introduction of extra copies of the A26 gene by plasmid transfection, and this strategy was used in some later experiments.

A26 and A27 physically interact. A physical association between A26 and A27 could stabilize the former. This idea was supported by our finding of a 90-kDa complex, which reacted with antibodies to A26 and A27, when VACV WR MVs were analyzed by SDS-PAGE and Western blotting in the absence of reducing agent (Fig. 3A). Moreover, only faint bands corresponding to the A26 monomer and A27 multimers were detected under these conditions (Fig. 3A). After treatment of VACV WR MVs with reducing agent, A26 and A27 were resolved as 60- and 14-kDa polypeptides, respectively (Fig. 3A). Furthermore, when purified vΔA26 virions were analyzed, the 90-kDa band was absent and only multimeric forms of A27 were detected (Fig. 3A).

Taken together, the above data suggested that the 90-kDa band was comprised of an A26-A27 disulfide-bonded complex. Immunoprecipitation experiments were carried out to verify the A26-A27 interaction. To facilitate such experiments, DNA encoding a V5 epitope tag was fused to the C terminus of the A26L ORF, and the recombinant vA26V5 was clonally isolated. Purified vA26V5 and control VACV WR virions were treated with 1% NP-40 to release membrane-associated proteins and incubated with V5 antibody that was conjugated to agarose beads. The unbound and bound proteins were resolved by SDS-PAGE under nonreducing conditions and detected by

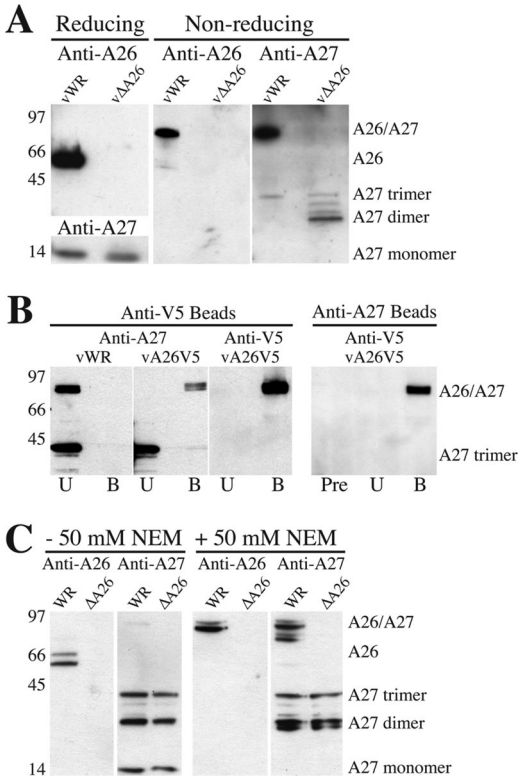

FIG. 3. A26 and A27 form an SDS-stable complex. (A) Western blot analysis of virions. Virions from cells infected with VACV WR (vWR) and vΔA26 were purified by sucrose gradient sedimentation, dissociated with LDS in the presence or absence of reducing agent, and subjected to SDS-PAGE. The blots were probed with antibody to A26 and then stripped and reprobed with antibody to A27. The positions of monomeric and oligomeric forms of A27, monomeric A26, and A26/A27 complex are indicated. (B) Immunoaffinity purification of A26/A27 complex. Purified virions from cells infected with VACV WR or vA26V5 MV were treated with 1% NP-40 detergent and incubated with antibody to V5 or A27 immobilized on protein A beads. Bound (B) and unbound (U) fractions were resolved by SDS-PAGE under nonreducing conditions and probed successively with antibody to A27 and V5 (left) or with antibody to V5 (right). Pre, eluate from protein A-agarose beads unconjugated to antibody. (C) Western blot of cell lysates. Cells infected with VACV WR or vΔA26 were harvested after 24 h and lysed in the presence (+) or absence (−) of 50 mM NEM. The proteins were resolved by SDS-PAGE under nonreducing conditions, and Western blots were probed with antibody to A26 and A27. In each panel, the numbers on the left are the masses in kDa of marker proteins.

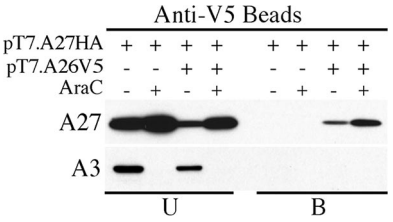

FIG. 4. A26 and A27 interact in the absence of other viral late proteins. Cells were infected with a recombinant VACV that expresses bacteriophage T7 RNA polymerase in the presence (+) or absence (−) of AraC and transfected with plasmids encoding epitope-tagged A26 (pT7.A26V5) and A27 (pT7.A27HA) regulated by T7 promoters as indicated. After approximately 18 h, the cells were harvested, and the lysates were incubated with antibody to V5 attached to beads. The unbound (U) and bound (B) fractions were analyzed by SDS-PAGE and Western blotting with antibody to HA and the VACV A3 protein.

Western blotting with anti-A27 and -V5 antibodies. When the unbound fraction from the control VACV WR virions was probed with antibody to A27, the major bands corresponded to the 90-kDa complex and trimeric A27; no bands were detected in the "bound" fraction because of the absence of the V5 tag (Fig. 3B). In contrast, the A27 antibody reacted with the 90-kDa complex in the bound fraction from vA26V5 virions, and only excess trimeric A27 was in the unbound fraction (Fig. 3B). The 90-kDa species was detected in the bound fraction when the blot was stripped and reprobed with anti-V5 antibodies, confirming that it was an SDS-stable complex of A26 and A27 (Fig. 3B). When the reciprocal experiment was conducted by incubating NP-40-treated vA26V5 virions with A27 antibody-conjugated agarose beads, the 90-kDa complex was detected by antibody to V5 only in the bound fraction (Fig. 3B). Taken together, these data indicate that A26 was physically associated with A27 in MVs. The Western blotting data obtained with VACV WR indicated that the majority of A26 and A27 proteins were present as a complex (Fig. 3A). The presence of substantial amounts of trimeric A27 in vA26V5 MVs indicated that the tagged A26V5 was present in smaller amounts than A27, possibly due to lower stability of the fusion protein (Fig. 3B). The A26-A27 complex was sometimes detected as a doublet, suggesting that A26 might be interacting with both A27 trimers and dimers.

Analysis of the A26-A27 complex in infected cell lysates. Initially we were perplexed to find that the 90-kDa A26-A27 complex was not detected when infected cell lysates were analyzed by SDS-PAGE and Western blotting in the absence of reducing agent; instead monomeric A26 and multimeric forms of A27 were resolved (Fig. 3C). Since the A26-A27 complex is held together by intermolecular disulfide bonds, we considered that these might be disrupted upon cell lysis by a process called disulfide interchange. For example, a free SH group on A27 could displace the disulfide bond between A26 and A27. An established way of preventing disulfide interchange is by adding NEM, which reacts with free sulfhydryl groups, prior to cell lysis. When NEM was added in this way, the 90-kDa complex was visualized by probing Western blots with antibodies to A26 and A27 (Fig. 3C). There was still excess A27 in the cytoplasm, as shown by the A27 trimers and dimers, but now there was no free A26. Therefore, it seems likely that the disulfide bond linking A26 and A27 was disrupted by disulfide interchange in the absence of NEM. This interchange may be catalyzed by some component in the cell extract in the presence of excess A27, since NEM was not needed to preserve the covalent A27-A26 complex isolated from purified virions.

A26-A27 complex formation is independent of other viral late proteins. A26 and A27 have late promoters and are therefore synthesized following viral DNA replication. The following scheme allowed the synthesis of A26 and A27 in the absence of DNA replication and other late proteins. Cells were (i) untreated or treated with AraC, an inhibitor of DNA replication to prevent late-gene expression; (ii) infected with vTF7.3, a recombinant VACV that expresses the T7 RNA

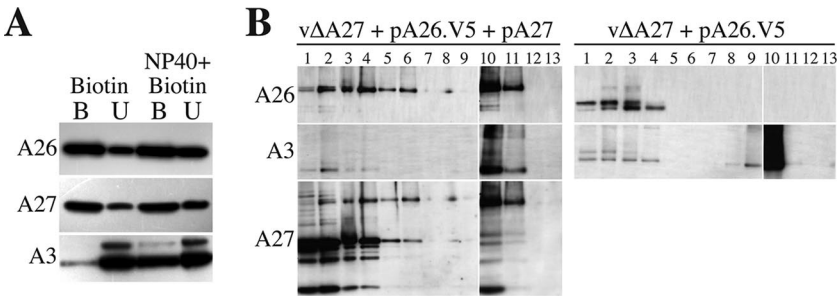

FIG. 5. A26 is anchored to the MV membrane surface by A27. (A) Biotinylation of A26 and A27 in intact virions. Sucrose gradient-purified virions from cells infected with VACV WR were treated with sulfo-NHS-SS-biotin and allowed to bind to NeutrAvidin beads. Bound (B) and unbound (U) fractions were analyzed by Western blotting with antibodies that recognized A26, A27, and A3. As a positive control, purified virions were treated with NP-40 prior to biotinylation. (B) Incorporation of A26 into purified virions. Cells were infected with vΔA27 and transfected with pA26V5 with or without pA27. After approximately 24 h, the cells were harvested and virions were purified by sedimentation through sucrose cushions and CsCl gradient sedimentation. Fractions (1 ml) were collected from the top and analyzed by Western blotting using antibodies that recognized A26V5, A27, and A3.

polymerase under an early promoter; and (iii) transfected with pT7.A26V5 or pT7.A27HA, plasmids that have epitope-tagged copies of A26 and A27, respectively, regulated by a T7 promoter. The cells were lysed 18 h after infection and incubated with beads that were conjugated with antibody to the V5 epitope tag on A26. The unbound and bound fractions were treated with reducing agent, resolved by SDS-PAGE, and analyzed by Western blotting with antibody to the HA tag of A27. Comparison of the unbound and bound fractions indicated that A27HA was bound to A26V5 in the absence or presence of AraC (Fig. 4). Furthermore, there was no nonspecific binding of A27HA to the V5 antibody in the absence of A26V5 expression. The higher expression of A27HA in the presence of AraC is probably due to the absence of competition by late mRNAs. There was also higher expression of A26V5 in the presence of AraC (data not shown). The inhibition of late-protein synthesis by AraC was demonstrated by Western blotting with antibody to the A3 protein.

A26 is anchored to the MV membrane surface by A27. Since A27 is located on the exterior of the MV membrane, a similar location for A26 seemed likely. Indeed, previous studies had shown that a protein called 4c or P4c, which probably corresponds to A26, could be labeled with iodine and released from purified virions by treatment with a nonionic detergent (18, 26). To confirm by another method that A26 was located on the MV surface, we carried out labeling experiments with a membrane-impermeable biotinylation reagent. Purified VACV WR virions were treated with sulfo-NHS-SS-biotin in the absence or presence of NP-40 detergent, which disrupts the MV membrane and exposes core proteins. Samples were incubated with NeutrAvidin beads to bind biotinylated proteins and then eluted with buffer containing dithiothreitol. The unbound and bound fractions were analyzed by SDS-PAGE, and the Western blot was successively probed with antibodies to A26 and A27. A26 was predominantly present in the bound fraction, similar to A27 (Fig. 5A). The A3 core protein was almost entirely in the unbound fraction, suggesting that the MV particles were intact and that the biotinylation reagent could not penetrate the viral membrane. The failure to biotinylate the A3 protein was not due to intrinsic nonreactivity, since A3 was biotinylated after disruption of the MV membrane with NP-40 and bound to NeutrAvidin beads (Fig. 5A).

In our initial experiment (Fig. 1), the absence of A26 in MVs produced in cells infected with vΔA27 could have been due mainly to the instability of A26. It was important, therefore, to determine whether A27 was also required for the association of A26 with MVs. As mentioned above, we found that the amount of A26 in cells infected with vΔA27 could be increased by introduction of extra copies of the A26 gene by transfection. Cells were infected with vΔA27 and cotransfected with the plasmid pA26V5, which expresses a V5 epitope-tagged A26, either alone or together with the plasmid pA27, which expresses A27. Virions were purified from the cell lysate by two successive sedimentations through a 36% sucrose cushion, followed by CsCl gradient centrifugation. Gradient fractions were analyzed by SDS-PAGE under nonreducing conditions and Western blotting. In the samples from the cells transfected with the two plasmids, both A26V5 and A27 were detected in a 90-kDa band in fractions 10 and 11 with the A3 core protein, corresponding to the expected MV density of ~1.27 g/ml (Fig. 5B). Additional A26V5 and A27 were present in the first several fractions near the top of the gradient. When A27 was not expressed, then A26V5 was present in the upper fractions as a 60-kDa protein and was absent from the lower MV fractions containing the A3 core protein (Fig. 5B). Thus, these data indicate that the association of A26 in the MV membrane is dependent upon A27.

The A26-A27 complex is associated with A17 and A25. Affinity purification was carried out to determine whether additional proteins were noncovalently associated with the A26-A27 complex. We were particularly interested in A17, since it anchors A27 to the viral membrane (24). Cells were infected with vA26V5 or VACV WR, and lysates were incubated with antibody to V5 coupled to beads. The bound proteins were eluted and visualized by staining after SDS-PAGE (Fig. 6). Several bands were specific for cells infected with vA26V5, whereas a few others were nonspecific. The specific bands were excised, and A25, A26, A17, and A27 were identified. Based on the intensity of the stain, A25 and A17 appeared to be present in smaller amounts than A26 and A27 (Fig. 6). However, this

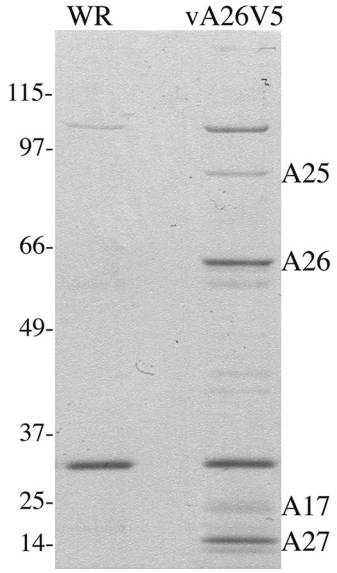

FIG. 6. Association of A17, A25, and A27 with A26 determined by mass spectrometry. Sucrose gradient-purified virions from cells infected with vA26V5 or VACV WR were treated with NP-40 and then bound to V5-specific antibody bound to beads. The bound proteins were resolved by SDS-PAGE and stained with Coomassie blue. The bands were excised, digested with trypsin, and analyzed by mass spectrometry. Bands identified as A17, A25, A26, and A27 are indicated.

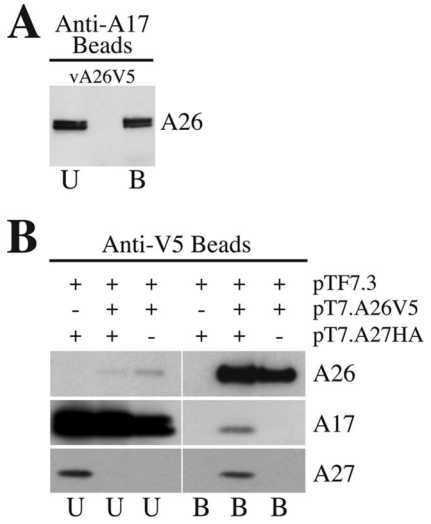

FIG. 7. Association of A26 with A17 was dependent on A27. (A) Immunopurification of A26 with immobilized A17. MVs purified from cells infected with vA26V5 were treated with NP-40 and incubated with antibody to A17 attached to protein A beads. Unbound (U) and bound (B) fractions were subjected to SDS-PAGE and Western blotting with antibody to the V5 epitope. (B) Immunoaffinity purification of A17 and A27 with A26. Cells were infected with vΔA27 and transfected with a plasmid expressing the T7 polymerase (pTF7.3) and plasmids expressing A26V5 (pT7.A26V5) and/or A27HA (pT7.A27HA) under the control of the T7 promoter. After approximately 24 h, the cells were harvested, lysed with NP-40, and incubated with antibody to V5 attached to beads. Proteins in the unbound and bound fractions were resolved by SDS-PAGE and analyzed by Western blotting with antibodies to V5, HA, and A17 as indicated.

may reflect the stability of the interactions rather than the stoichiometry of the complex. Proteins corresponding to other faint bands could not be identified by mass spectroscopy analysis.

Immunoprecipitation and Western blotting confirmed the association of A17 with the A26-A27 complex in virions. MVs purified from cells infected with vA26V5 were treated with NP-40 and then incubated with beads that were conjugated with antibody to A17. A26V5 was detected by Western blotting of proteins that were immunopurified with A17 antibody (Fig. 7A).

Association of A26 with A17 is mediated by A27. Since A27 is known to interact with A17 (24), the association of the latter with A26 could be indirect. Alternatively, A26 might also bind A17 directly. To investigate these alternatives, cells were infected with vΔA27 and transfected with combinations of plasmids expressing bacteriophage T7 RNA polymerase from a VACV promoter and A26V5 and A27HA from T7 promoters. When all three plasmids were transfected, beads coupled to V5 antibody pulled down A26V5, A27HA, and A17 (Fig. 7B). However, when the plasmid expressing A27HA was omitted, only A26V5 was captured. Thus, A27 was required for the interaction of A26 with A17.

The C terminus of A26 mediates interactions with A27. The C-terminal region of A26, from amino acids 441 to 472, shares 44% amino acid identity with the A27 C-terminal alpha-helical "anchoring domain" through which A27 mediates interactions with A17 and the MV particle (34). Two plasmids were constructed: pA26ΔNV5 expressed the C-terminal 270 to 500 amino acids and included the region of homology with A27, whereas pA26ΔCV5 coded for amino acids 1 to 409, excluding the region of homology to A27 (Fig. 8A). Cells were infected with vΔA26 and transfected with either empty plasmid or plasmids expressing full-length A26V5 or the deletion mutants. After 18 h, the cells were lysed with 1% NP-40 and the soluble extract was incubated with anti-V5-conjugated beads. The unbound and bound fractions were resolved by SDS-PAGE under reducing conditions and probed with antibodies against A26, A27, and A17. Full-length A26 and the C-terminal 220-amino-acid fragment from pA26ΔNV5 pulled down A27 and A17 (Fig. 8B). Neither A27 nor A17, however, was present in the bound fractions from cells expressing the C-terminal deleted form of A26 from pA26ΔCV5 (Fig. 8B). The region from amino acids 270 to 500 contains three cysteines, at least one of which may be involved in the disulfide bond with A27.

Deletion of A26 does not increase EV formation. The discovery that A26 is present in MVs but not EVs led to a suggestion that A26 negatively regulates wrapping (33). Our finding that A26 interacted with A27, which is required for wrapping, could have provided a mechanism for such a role. If this hypothesis were correct, then deletion of the A26L ORF should increase the number of EVs. RK-13 and HeLa cells were infected with either vΔA26 or VACV WR, and the cells and media were harvested separately after 48 h. The deletion of A26L did not increase the amount of EV either released naturally into the medium (Fig. 9A) or liberated from the surface of the cell with trypsin (Fig. 9B). Furthermore, there was no discernible difference in plaque size or formation of

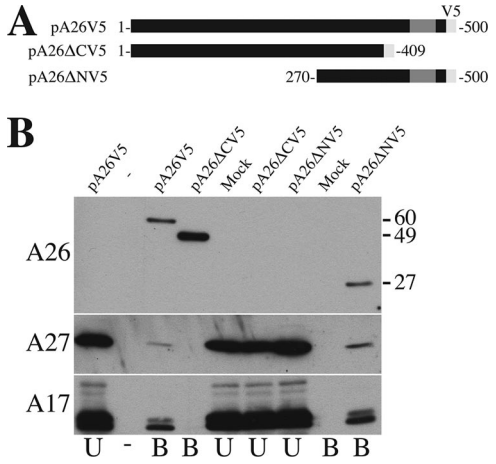

FIG. 8. The C terminus of A26 interacts with A27. (A) Schematic drawings of truncated A26 ORFs. pA26V5, pA26ΔCV5, and pA26ΔNV5 show the lengths of V5 epitope-tagged A26 plasmid inserts. Black shading, A26 sequence; dark gray, region of homology with A27; light gray, V5 epitope tag. A26 amino acid residues are numbered. (B) Western blots of immunoaffinity-purified A26-associated proteins. Cells were infected with vΔA26 and mock transfected or transfected with pA26V5, pA26ΔCV5, or pA26ΔNV5. Lysates were incubated with antibody to V5 coupled to beads, and the unbound and bound fractions were analyzed by SDS-PAGE and Western blotting with antibody to V5, A17, and A27. The positions and masses in kDa of marker proteins are shown on the right.

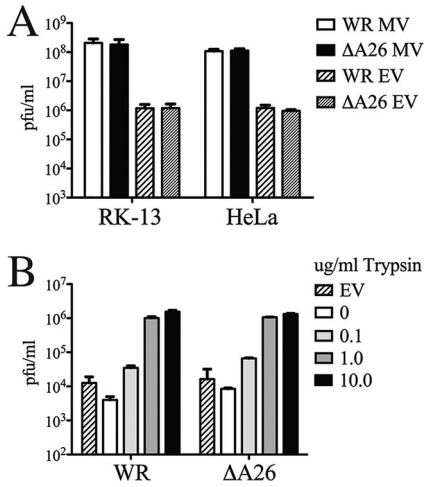

FIG. 9. Intra- and extracellular virus production. (A) RK-13 and HeLa cells were infected with VACV WR or vΔA26 at a multiplicity of infection of 0.1 PFU per cell. After 48 h, the media were collected and the intra- and extracellular virus titers were determined. (B) The indicated concentrations of trypsin were used to release EVs from the surfaces of cells infected with VACV WR and ΔA26 for 24 h. Virus titers were determined by plaque assay. Error bars are shown.

satellite plaques due to release from EVs from RK-13 cells infected with vΔA26 compared to wild-type virus (not shown).

DISCUSSION

A26 is an interesting protein that appears to have roles in attaching MVs to the cell surface (3) and incorporating MVs into ATIs formed by some orthopoxviruses (19). The present study was initiated following our serendipitous observation that A26 was missing from MVs formed by an A27L deletion mutant. Further studies indicated that A27 was required for the stability of A26, as well as for its incorporation into MVs. The relationship between A26 and A27 was explained by the finding that the two proteins formed an SDS-stable complex in the absence of reducing agent. The complex migrated as a band of about 90 kDa, likely consisting of A26 (58 kDa) disulfide bonded to A27 (12.6-kDa) trimers or dimers. Moreover, nearly all of the A26 and A27 proteins in wild-type MVs were in the complex. It was surprising, therefore, that this complex was not generally recognized during analysis of virion proteins, though it likely corresponds to the heterodimer of 54- and 16-kDa polypeptides described by Ichihashi (14). We had trouble detecting the A26-A27 complex in cytoplasmic extracts until we realized the importance of adding NEM to prevent disulfide interchange after lysis, which is probably mediated by excess A27 and perhaps other cytoplasmic proteins. This phenomenon may provide an explanation for the failure of others to observe the A26-A27 complex. Further studies indicated that no additional viral late proteins were required for the interaction of A26 and A27. Coimmunoprecipitation experiments indicated that the C-terminal segment of A26 interacted with A27. Interestingly, part of that region shares sequence homology with A27. These data are consistent with an observation that the C-terminal 75 amino acids of A26 are required for anchoring the protein to the MV particle, although an interaction with A27 was not proposed at the time (3).

The A26-A27 complex also associated noncovalently with the A17 and A25 proteins. A17, a transmembrane protein, had previously been shown to interact with A27 (24), and our study indicated that the association of A26 with A17 was indirect and dependent on A27. The inability of A26 to interact directly with A17 (or any other integral membrane protein) explains why A27 is needed for incorporation of A26 into MVs. Biotinylation studies confirmed that A26 and A27 were exposed on the surface of the MV membrane.

The finding of a multiprotein A17, A25, A26, and A27 complex has interesting implications. Previous studies have shown that the formation of the ATI is dependent on a full-length ortholog of the truncated A25 protein and that A26 is needed for the embedding of virions within it (19). The role of A26 in this process has not been elucidated, although a role in retrograde transport on microtubules was suggested. The interaction of A26 with A25, shown here, suggests a direct role of A26 in attaching virions to the ATI. It is thought that the inclusions protect virions in a hostile environment and allow animal-to-animal transmission. However, a reduction in EVs, which are important for cell-to-cell spread, appears to be a consequence of virion occlusion, since it results in smaller plaques (15, 16). Ulaeto and coworkers (33) suggested a related role for A26 in orthopoxviruses that do not make ATIs. Primarily because A26 was found in MVs but not EVs, they suggested that A26 might negatively regulate wrapping. Our finding that A26 interacts with A27, which is required for MV wrapping (25), made the hypothesis that A26 modulates EV formation even more attractive. Nevertheless, we did not discern a change in the ratio of EVs to MVs when the A26L ORF was deleted. It remains possible, however, that the number of MVs lacking A26 was

not limiting for wrapping in the two cell lines that were tested but that the situation would be different with other cells, other VACV strains, or other incubation conditions. We did find a reduction in EV production when A26 was overexpressed, but this was correlated with a reduction in the expression of other viral proteins, including A27, and was therefore difficult to interpret (our unpublished data).

A26 appears to have a role in cell attachment, as it was shown that A26 binds laminin, an extracellular matrix protein, and soluble laminin partially blocks MV binding to cells (3). Chiu et al. (3) reported that virions lacking A26 are still infectious, and we confirmed this more directly with an A26L deletion mutant. Retention of infectivity was not surprising, since VACV encodes other proteins that mediate cell attachment by binding glycosaminoglycans (4, 13). Since A27 is one of the three glycosaminoglycan-binding proteins, there could be functional significance to the physical association of two membrane attachment proteins.

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