The A33-Dependent Incorporation of B5 into Extracellular Enveloped Vaccinia Virions is Mediated Through an Interaction Between Their Lumenal Domains

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Running title: A33-B5 interaction during vaccinia virus infection

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ABSTRACT

There are two mechanisms for the incorporation of B5 into the envelope of extracellular virions produced by orthopoxviruses, one that requires A33 and one that does not. We have hypothesized that the A33-dependent mechanism requires a direct interaction between A33 and B5. In this study, chimeric constructs of A33 and B5/B5-GFP were used to show that the two proteins interact through their lumenal domains and show that the coiled-coil domain of B5 is sufficient for an interaction with A33. Furthermore, our experiments reveal that a transmembrane domain, not necessarily its own, is requisite for the lumenal domain of B5 to interact with A33. In contrast, the lumenal domain of A33 is sufficient for interaction with B5. Furthermore, the lumenal domain of A33 is sufficient to restore proper localization of B5-GFP in infected cells. Taken together, our results demonstrate that the lumenal domains of A33 and B5 interact and that the interaction is required for the incorporation of B5-GFP into extracellular virions, whereas the incorporation of A33 is independent of B5. These results suggest that viral protein incorporation into extracellular virions is an active process requiring specific protein-protein interactions.
INTRODUCTION

Remarkably, orthopoxviruses produce two infectious forms that are morphologically and antigenically distinct (1, 35). Viral replication occurs entirely in the cytoplasm of infected cells in a specialized area known as the viral factory, where the first form of infectious virions, termed intracellular mature virions (IMV), is produced (7, 26). IMV represent the majority of progeny virions and are only released if the cell is lysed. A subset of IMV is transported along microtubules to the site of wrapping and obtain an additional double membrane envelope derived from the trans-Golgi network or early endosome (15, 40, 45). These wrapped virions are termed intracellular enveloped virions (IEV). IEV are transported along microtubules to the plasma membrane and released from the cytoplasm by fusion of their outermost membrane with the plasma membrane (13, 17, 33, 52). Virions retained on the cell surface are termed cell-associated enveloped virions (CEV). Some CEV are propelled away from infected cells by actin tails, a process that requires the viral A36 protein (16, 37, 44, 56). When CEV are released from the cell surface, they become extracellular enveloped virions (EEV). CEV are required for efficient cell-to-cell spread (43, 52) while EEV are required for long-range dissemination of virus (1, 30). CEV and EEV are collectively termed extracellular virions (EV) (27).

Eight proteins encoded by vaccinia virus, A33 (36), A34 (8), A36 (48), A56 (42), B5 (11, 55), F12 (47, 58), F13 (2), and K2 (46, 49), have been shown to be specific for IEV. Deletion of the gene that encodes any of these proteins, with the exception of A56 and K2, results in a small plaque phenotype, implying that these proteins have a role in EV morphogenesis and/or infectivity. Several interactions amongst these proteins have
been reported and it appears that these interactions are required for the proper complement of viral proteins in the envelope of progeny EV (6, 10, 22, 29, 31, 32, 39, 54, 57). Among these proteins, the integral membrane protein A33 has been reported to interact with both A36 (52, 57) and B5 (3, 31). A36 requires an interaction with A33 for incorporation into the outer IEV membrane (57), suggesting that A33 has the ability to target proteins to the EV envelope. B5 has been shown to target to virion membranes in the absence of A33 (5, 31). It has been reported that the incorporation of A33 into wrapped virions requires an interaction with B5 (31). Subsequently, we reported that chimeric B5-GFP requires an interaction with A33 for its incorporation into EV (5). This implies that there are two mechanisms for the incorporation of B5 into wrapping membranes, one that is dependent on A33. The other mechanism appears to be inhibited by the addition of GFP to B5. In an effort to clear up this controversy and determine the dependency of the B5-A33 interaction for proper intracellular trafficking and virion incorporation, we have characterized the A33-B5 interaction in detail. Our studies show that the trafficking and EV incorporation of A33 is independent of B5. In addition, we have mapped the region of interaction to the luminal domains of both proteins and show that the luminal domain of A33 is sufficient to properly target B5-GFP and mediate its incorporation into EV. These results, in conjunction with our companion paper (4), support the conclusion that incorporation of B5 into the EV membrane is an active process that is normally mediated through an interaction with A33.

MATERIALS AND METHODS

Cells and viruses.
HeLa and RK13 cells were maintained as previously described (50). Construction of vB5R-GFP (53), vB5R-GFP/∆A33R (5), vTF7.3 (9), and v∆B5R (55) has been described previously.

**Plasmid constructs.**

Construction of pB5-GFP, pB5/cc/G/Δ-GFP (51), and pA33R-HA (3) has been described previously. To construct p∆/cc/B5/Δ-GFP, overlapping primer pairs were designed to amplify the coding sequence for residues 1-28 and 242-303 of B5. These fragments were joined by a second PCR and subsequently cloned using engineered 5′-HindIII and 3′-NcoI sites in place of the full length B5 coding sequence in pB5-GFP. A plasmid containing the coding sequence of influenza virus A/Chicken/Nanchang/3-120/01 neuraminidase (NA) was a gift from Toru Takimoto at the University of Rochester Medical Center. A chimeric A33 that has the cytoplasmic tail domain deleted and the transmembrane domain replaced with that of influenza virus Nanchang NA (∆/NA/A33R-HA) was constructed by two-step overlapping PCR. For expression, the coding sequence of ∆/NA/A33R-HA was inserted under control of the T7 promoter in pcDNA3 (Invitrogen) (p∆/NA/A33R-HA). To construct B5 lacking the transmembrane and cytoplasmic tail domains (pB5R°/cc), the sequence encoding amino acid residues 1 to 275 followed by a stop codon was amplified by PCR and inserted under control of the T7 promoter in pcDNA3 (Invitrogen). To construct A33-HA lacking the transmembrane and cytoplasmic tail domains (A33R-HA°/LD), the sequence encoding amino acid residues 61 to 185 followed by an HA epitope tag and a stop codon, which was preceded by the signal peptide sequence of vesicular stomatitis virus G (Indiana strain) (VSVG), was amplified by PCR and inserted under control of the T7 promoter in pcDNA3 (Invitrogen).
to yield pA33R-HA<sup>LD</sup>. To place the coding sequence of A33R-HA<sup>LD</sup> under the control of the normal A33R promoter, two separate PCR reactions were performed. The first reaction amplified the ~500 bp upstream region of A33R, which contains the A33R promoter. The second reaction amplified the coding sequence of the soluble form of A33 with the HA epitope tag. The two resulting products were joined by an overlapping PCR and this product was inserted into pCR2.1 (Invitrogen) to yield pLF A33R-HA<sup>LD</sup>. The coding sequence of A33R-HA plus ~500 bp of upstream sequence, which would include the normal A33R promoter, was removed out of pLF A33R-HA-118 (5) with ApaI and HindIII and inserted into pCR2.1 that had been digested in the same way. The resulting plasmid, pLF A33R-HA, was sequenced to verify its integrity. To construct pVSVG-GFP, the coding sequences of VSVG and enhanced GFP were amplified by PCR. PCR fragments were annealed using an overlapping PCR. The resulting PCR product was inserted into pCR2.1 (Invitrogen) and subcloned into pcDNA3 (Invitrogen) that had been digested with HindIII and XhoI. Oligos were designed to remove the coding sequence of GFP and add the coding sequence for a Strep-tag II (41) epitope followed by a stop codon for constructs B5-GFP, B5/cc/B5/Δ-GFP (51), and Δ/cc/B5/Δ-GFP using PCR. The resulting products, which contained the coding sequence for B5-Strep, B5-cc/B5/Δ-Strep, and Δ/cc/B5/Δ-Strep, respectively, were inserted into pCR2.1 (Invitrogen) and subcloned into pcDNA3 (Invitrogen) using standard cloning techniques. Similarly, an oligo was designed to add the coding sequence for a Strep-tag II after the residue 482 of the transmembrane domain of VSVG. This oligo was used with the overlapping oligos described above, which remove residues 29-241 of B5 in construct pA/cc/B5/Δ-GFP, in a two-step PCR to remove the lumenal domain residues 29-241 and replace the coding
sequence of GFP with the coding sequence for the Strep-tag II epitope followed by a stop
codon of construct B5/cc/G/Δ-GFP. The resulting product contained the coding sequence
for Δ/cc/G/Δ-Strep. It was inserted into pCR2.1 (Invitrogen) and subcloned into pcDNA3
(Invitrogen) using standard cloning techniques. All constructs were verified by
sequencing.

**Immunofluorescence microscopy.**

HeLa cells grown on glass coverslips were infected with either vB5R-GFP or vΔB5R at a
multiplicity of infection (MOI) of 1.0. For *in vivo* trans-complementation, HeLa cells
infected with vB5R-GFP/ΔA33R at a MOI of 1.0 were transfected with either pLF A33R-
HA or pLF A33R-HA LD or mock transfected. The next day, cells were fixed with 4%
paraformaldehyde in phosphate-buffered saline (PBS). For intracellular staining, fixed
cells were permeabilized with 0.1% Triton X-100 in PBS. Fixed or fixed and
permeabilized cells were incubated with an anti-A33 MAb (10F10), kindly provided by
Jay Hooper (18), or rabbit anti-HA antibody (Sigma), followed by Texas Red-conjugated
donkey anti-mouse or anti-rabbit antibody, respectively (Jackson ImmunoResearch
Laboratories). DNA in the nuclei and viral factories were stained with either 4’, 6’-
diamidino-2-phenylindole dihydrochloride (DAPI) or Hoechst as described previously
(5). Cells were visualized and imaged as previously described (50). Images were
minimally processed and pseudo-colored using Adobe Photoshop software (Adobe
Systems).

**Immunoprecipitation and Western blotting.**

HeLa cells infected with vTF7.3 at a MOI of 5.0 in the presence of 40 µg/ml of cytosine
arabinoside (AraC) (Sigma) were transfected with various plasmids containing the coding
sequence of genes under control of the vaccinia virus T7 promoter at 2 h PI. The same
amount of each construct was used in every transfection and a total of 1 µg of total DNA
was used for each transfection. In experiments where the total amount of constructs did
not equal 1 µg the difference was made up using pcDNA3. Transfection media was
removed at 4.5 h post-transfection and replaced with media containing 25 µCi/ml of ³⁵S-
Met/Cys (Perkin Elmer). For co-immunoprecipitation during infection, HeLa cells were
infected with vB5R-GFP/ΔA33R at a MOI of 5.0 and transfected with either pLF A33R-
HA or pLF A33R-HA/DD or mock transfected. The following day, cells were harvested by
scrapping, washed once in PBS, and lysed in radioimmunoprecipitation assay (RIPA)
buffer (0.5x PBS, 0.1% sodium dodecyl sulfate, 1% Triton X-100, 1% NP-40, and 0.5%
sodium deoxycholate) containing protease inhibitors as previously described (5).
Immunoprecipitation was performed using an anti-HA MAb (Santa Cruz Biotechnology)
as previously described (10). Proteins were resolved on 4-12% gradient or 12%
acrylamide gels (Invitrogen) and detected by autoradiography or Western blots. For
Western blots, proteins were transferred to nitrocellulose membranes. Membranes were
incubated with a horseradish peroxidase (HRP)-conjugated anti-GFP antibody
(Rockland), HRP-conjugated anti-HA antibody (Roche), an anti-GFP MAb (Covance),
anti-HA MAb (Roche), or anti-Strep-Tag II MAb (Novagen). Unconjugated antibodies
were followed with an appropriate HRP-conjugated anti-mouse or anti-rat antibody,
(Jackson ImmunoResearch Laboratories). Bound antibodies were detected using
chemiluminescent reagents (Pierce) following manufacturer’s instructions.

Analysis of EEV.
RK_{13} cells were infected with vB5R-GFP, vΔB5R, or vB5R-GFP/ΔA33R at a MOI of 10.0. 4 h PI, media was replaced with media containing ^{35}S-Met/Cys (Perkin Elmer). The next day, radiolabeled virions released into the media were purified through a 36% sucrose cushion. The resulting viral pellets were lysed in RIPA buffer as described above. EEV lysates were equilibrated by scintillation counting and equal counts were subjected to immunoprecipitation with an anti-A33 MAb. Antibody-protein complexes were pulled down as described previously (10). Immunoprecipitated proteins were analyzed by SDS-PAGE and detected by autoradiography.

**Immunoelectron microscopy.**

RK_{13} cells were infected with either vB5R-GFP or vΔB5R at a MOI of 10.0. The next day, virions released into the media were purified as described above. Purified virions were adsorbed to Formvar-coated nickel grids and immunostained with either an anti-B5 MAb or anti-A33 MAb, followed by 18 nm colloidal gold-conjugated goat anti-rat or anti-mouse, respectively (Jackson ImmunoResearch Laboratories). Virions were negatively stained and visualized using a Hitachi 7650 TEM with a Gatan 11 Megapixel digital camera.

**RESULTS**

**B5 is not required for proper subcellular localization of A33.**

B5 is a type I integral membrane protein that has been shown to localize to EV in the absence of A33 (5, 21, 31). It has been reported that the interaction between A33 and B5 is required for the incorporation of A33 into EV (31). In contrast, a chimeric version of B5 (B5-GFP) was shown to require A33 for incorporation (5), indicating that there are two mechanisms for B5 incorporation, one that is dependent on A33 and one that is not.
To investigate the dependency of these two proteins on each other for proper intracellular trafficking and virion incorporation, we first examined if A33 was properly localized during infection in the absence of B5. In cells infected with vB5R-GFP, A33 colocalized with B5-GFP at the site of wrapping, at the cell vertices, and on virion-sized particles (VSPs), which are three characteristic hallmarks of both B5 and B5-GFP in infected cells (Fig. 1A) that have been seen previously (53). Moreover, B5-GFP provides a convenient tool for studying the A33-dependent pathway because B5-GFP is dependent on A33 for incorporation whereas B5 is not. Therefore, B5-GFP was used in these studies. It should be noted that the localization pattern of B5-GFP observed in cells infected with vB5R-GFP is almost identical to the B5 localization pattern seen in cells infected with the parental virus, WR (5), indicating that fusion of GFP to B5 does not alter the proper localization of B5 in the presence of all other viral proteins. Similarly, in cells infected with a virus that has B5R deleted (vΔB5R), A33 localized at the site of wrapping, at the cell vertices, and on VSPs in a pattern similar to that seen in cells infected with vB5R-GFP (Fig. 1A), demonstrating that proper subcellular localization of A33 is independent of B5. To look for A33 on CEV in the absence of B5, cells infected with vΔB5R were stained without permeabilization using an anti-A33 MAb. In cells infected with vB5R-GFP, A33 was found on the plasma membrane and concentrated with GFP-labeled VSPs, (inset box Fig. 1B). Importantly, the site of wrapping was not stained, confirming that the cells were unpermeabilized. In the absence of B5, a similar staining pattern was seen with A33 on the plasma membrane and on VSPs (Fig. 1B), although the number of VSPs was greatly reduced. Thus, the proper subcellular targeting of A33 is independent of B5.

**A33 is efficiently incorporated into EEV in the absence of B5.**
The previous results show that the cellular localization of A33 is unchanged in the absence of B5 and suggest that A33 may be incorporated into EV. To determine if the incorporation of A33 into EV is independent of B5, we looked for A33 in purified EEV released by RK13 cells infected with vΔB5R. We purified radiolabeled virions released by RK13 cells infected with vB5R-GFP, vΔB5R, or vB5R-GFP/ΔA33R. Purified virions were lysed and equal amounts of radioactivity were subjected to immunoprecipitation with an anti-A33 MAb. A band of the predicted size for A33 was immunoprecipitated from EEV released by cells infected with either vB5R-GFP or vΔB5R but not from EEV released by cells infected with vB5R-GFP/ΔA33R (Fig. 2A), indicating that the incorporation of A33 into extracellular virions is independent of B5. The examination of equilibrated EEV lysates by SDS-PAGE showed that approximately equal amounts of protein were used in the assay (Fig. 2A).

To verify our biochemical analysis, we performed immunoelectron microscopy on purified EEV to visualize the presence of A33 on extracellular virions. Purified EEV released from cells infected with either vB5R-GFP or vΔB5R were immunolabeled with either an anti-B5 or anti-A33 MAb, followed by colloidal gold-conjugated goat anti-rat or anti-mouse antibody, respectively. B5-GFP and A33 were readily detected on EEV released from cells infected with vB5R-GFP (Fig. 2B). A33, but not B5, was detected on EEV released from cells infected with vΔB5R (Fig. 2B), supporting our biochemical analysis. Taken together, our data show that B5 is not required for proper subcellular localization and incorporation of A33 into wrapped virions.

**A33 and B5 interact through their luminal domains.**
The results from the above experiments show that the localization and incorporation of A33 into EV is independent of B5. We have shown that A33 interacts with B5-GFP (3) and that the incorporation of B5-GFP into IEV/EV requires A33 (5). Therefore, we hypothesized that the interaction between A33 and B5-GFP was necessary for B5-GFP incorporation into EV. We next wanted to map the domains in A33 and B5 that are involved in interaction using co-immunoprecipitation (CoIP). B5 and A33 are type I and type II integral membrane proteins, respectively (Fig. 3A) (21, 36). Chimeras were generated in which the various domains of B5 and A33 (lumenal, transmembrane, and cytoplasmic tail, LD, TMD and CT, respectively) were replaced with the corresponding domains from analogous non-poxvirus proteins that do not interact (the G protein from VSV and the NA protein from influenza A virus, respectively). Diagrammatic representations of the chimeras constructed are depicted in Fig. 3A with each predicted domain (LD, TMD, and CT) depicted by a letter representing the protein it was derived from (B5, A33, G, and NA proteins) separated by a slash. For B5, the predicted coiled-coil (CC) is also included as VSVG is not predicted to encode such a structure. Interaction of the chimeras was tested for by co-expression using the vaccinia virus T7 expression system (9) in the presence of AraC, to inhibit viral late protein synthesis and reduce the production of endogenous untagged late proteins that may compete for interaction in our assay, followed by CoIP. As expected, a band corresponding to full-length B5-GFP was co-immunoprecipitated with A33-HA, confirming the interaction between B5 and A33 (Fig. 3B) (3). Similarly, a chimera that has the lumenal domain of A33 and the transmembrane domain of influenza NA (∆NA/A33-HA) also interacted
with B5-GFP, suggesting that the two proteins interact through their luminal domains (Fig. 3B).

Next, we replaced the transmembrane domain of B5 with the transmembrane domain from VSVG to test the ability of the luminal domain to interact with A33. This new construct (B5/cc/G/Δ-GFP) was able to interact with both full-length A33-HA and a construct that only has the luminal domain of A33, Δ/NA/A33-HA, confirming that an interaction occurs between their luminal domains (Fig. 3B). Amino acid residues 236 to 276 of B5 are predicted to form a coiled-coil structure. These structures are known to be involved in protein-protein interactions (19). A truncated version of B5R-GFP that only encodes the predicted coiled-coil and transmembrane domains of B5 fused to GFP, pΔ/cc/B5/Δ-GFP, was tested to determine its ability to interact with A33. Δ/cc/B5-GFP was co-immunoprecipitated with A33-HA (Fig. 3B), indicating that much of the luminal domain and the cytoplasmic tail of B5 are not required for interaction with A33. Taken together, our results suggest that the coiled-coil domain of B5 interacts with the luminal domain of A33. Expression of all constructs was verified by Western blots using appropriate antibodies (Fig. 3C).

The luminal domain of A33 is sufficient for interaction with B5.

The above result indicates that A33 and B5 interact through their luminal domains. To test if the luminal domains are sufficient for interaction, constructs were made that express the predicted luminal domains of B5 and A33-HA (pB5LD/cc and pA33R-HA LD, respectively) (Fig. 4A). Using the same T7 expression system as above, the new constructs were tested for their ability to interact using CoIP. A33-HA LD brought down a band of the predicted size for the full-length B5-GFP (Fig. 4B), demonstrating that the
lumenal domain of A33 is sufficient for interaction. However, an interaction between
B5LDcc and either the full-length A33-HA or A33-HA LD was not detected (Fig. 4B),
suggesting that anchorage of B5 via a transmembrane domain is required for the
interaction to occur. Expression of B5LDcc was verified by Western blot using an anti-B5
MAb (Fig. 4C). Immunofluorescence microscopy indicated that the inability of the
soluble proteins to interact was not due to dissimilar subcellular localization (data not
shown).

Membrane anchoring of B5 is required for interaction with the lumenal domain of
A33.

We wanted to rule out the possibility that the transmembrane domain of B5 was required
for interaction with A33. Therefore, we tested if the lumenal domain of B5, including the
coiled-coil, fused to the transmembrane domain of VSVG (B5/ccc/G/Δ-GFP) could
interact with A33-HA LD using CoIP (Fig. 5A). A33-HA LD co-immunoprecipitated a band
corresponding to B5/ccc/G/Δ-GFP, indicating that the B5 transmembrane domain is not
required for the interaction but membrane anchoring by an unrelated transmembrane
domain is (Fig. 5B).

The coiled-coil domain of B5 is sufficient for interaction with A33. Cumulatively, all
of the previous results suggest that the coiled-coil region of B5 is sufficient for
interaction with A33. However, these experiments were conducted using B5 constructs
fused to GFP. Although it seems highly unlikely, we cannot rule out the possibility that
GFP was influencing the interaction with A33. To directly test the coiled-coil region of
B5 for interaction with A33, and to rule out any involvement of GFP, we made four new
constructs. All of the new constructs have a COOH-terminal Strep-tag II epitope in place
of GFP (Fig 6A) and were tested for interaction with A33-HA. As had been seen with their GFP containing counterparts, full length B5, a truncation missing the cytoplasmic tail, and a truncation missing the cytoplasmic tail in addition to the lumenal domain (B5-Strep, B5/cc/B5/Δ-Strep, and Δ/cc/B5/Δ-Strep, respectively) were co-immunoprecipitated with A33-HA (Fig. 6B). Several non-specific bands were detected in all of the samples, notably, the two at approximately 51 and 28 kDa likely represent the heavy and light chains, respectively, from the antibody used to perform the precipitation. A new construct that contains the predicted coiled-coil domain fused to the transmembrane domain of VSVG (Δ/cc/G/Δ-Strep) was also co-immunoprecipitated with A33-HA although at much reduced levels compared to B5-Strep. This reduction is most likely due to poor expression of the truncation (Fig. 6C). An additional, a faster migrating, band was detected in the cell extract and pull downs from cells that expressed Δ/cc/B5/Δ-Strep. We are unsure if this band was the result of cellular processing of the truncation, or of sample handling during analysis. Regardless, as VSVG is not known to interact with A33 (Fig. 3A), these results show that the coiled-coil domain of B5 is sufficient for interaction with A33.

The lumenal domain of A33 is sufficient for interaction with B5-GFP and its incorporation into extracellular virions during infection.

The above co-immunoprecipitations were carried out using the vaccinia virus T7 expression system in the absence of late viral protein synthesis and therefore, viral morphogenesis. The use of fusion proteins, while convenient for mapping interaction domains, may not reflect interactions that happen during a typical infection. Therefore, we wanted to determine if an interaction between the lumenal domain of A33 and B5-
GFP could be detected during infection. Cells were infected with a recombinant virus that has A33R deleted (vB5-GFP/ΔA33R) and transfected with plasmids that contained either full-length A33R-HA or A33R-HA<sup>LD</sup>, (pLF A33R-HA or pLF A33-HA<sup>LD</sup>, respectively) under the control of the natural A33R promoter so that they would be expressed during infection. Once again, B5-GFP provides a convenient tool for studying the A33-dependent pathway because B5-GFP is dependent on A33 for proper localization and EV incorporation. Both A33-HA and A33-HA<sup>LD</sup> co-immunoprecipitated B5-GFP (Fig. 7A), indicating that the lumenal domain of A33 interacted with B5-GFP during infection. A non-The reduced amount of B5-GFP co-immunoprecipitated with A33-HALD compared to A33-HA is likely the result of the reduced expression of A33-HA<sup>LD</sup> (Fig. 7A).

In the absence of A33, B5-GFP is mis-targeted during infection and EV are produced that do not contain detectable amounts of B5-GFP (5). Providing full-length A33 in trans restores proper localization of B5-GFP to VSPs in the cytoplasm and cell vertices (5). We wanted to determine if the lumenal domain A33 was sufficient to restore localization of B5-GFP to VSPs. Therefore, we repeated the assay and looked at the localization of B5-GFP using fluorescence microscopy. As shown in Fig. 6B, VSPs labeled with B5-GFP could be visualized in cells transfected with either the full-length A33-HA or A33-HA<sup>LD</sup>. In contrast, we were unable to detect B5-GFP labeled VSPs in the cytoplasm of cells that were mock transfected (Fig. 7B). Interestingly, the B5-GFP labeled VSPs produced in the presence of A33-HA<sup>LD</sup> did not stain with the anti-HA antibody (Fig. 7B). Taken together, our results indicate that the lumenal domain of A33 is sufficient for both the interaction with B5-GFP and to drive its incorporation into EV.
DISCUSSION

Proper glycoprotein composition of the nascent viral envelope is important for efficient production and release of infectious wrapped virus. Of the eight virus-encoded IEV-specific proteins, only F13 has been predicted to have an enzymatic activity (20). Therefore, we theorized that the other IEV-specific proteins have structural roles during morphogenesis and as such, the interactions amongst these proteins coordinate intracellular envelopment of IMV and assure proper protein composition. Interactions between IEV-specific proteins have been described for the coordinated incorporation of proteins into IEV: A33 and A36 (54, 56), A33 and B5 (3, 31), A34 and B5 (10, 32), A36 and F12 (22), and E2 and F12 (19). The purpose of the present study was to examine the previously described interaction between A33 and B5 (3, 31) and determine which protein is dependent on the other for IEV incorporation. It was reported that in the absence of B5, colocalization of A33 with DNA staining particles in the cytoplasm were not observed and concluded that the interaction between A33 and B5 was required for the incorporation of A33 into IEV (31). In contrast, when we looked directly at EV formed in the absence of B5, we were able to detect A33 (Fig. 1 and 2). Our results are supported by the findings of Rottger et al. (38). Using immunofluorescence microscopy, they reported that particles labeled with both A27 (an IMV-specific protein) and A33 were observed in the cytoplasm of infected cells in the absence of B5 (38). In addition, they found that actin tails were formed in the absence of B5 (38), a process that requires A33 for the incorporation of A36 into IEV (56). Our results demonstrate that A33 does not require B5 for incorporation into EV, but do not rule out the possibility that A33 is dependent on a different IEV-specific protein for its incorporation.
B5 has been reported to have a role in EV formation as deletion of the gene causes a reduction in EV production (12, 21, 55). Previous studies have shown that the cytoplasmic tail (25) and a large portion of the extracellular domain of B5 (14) are not required for EV formation, indicating an important role for the transmembrane domain and the coiled-coil structure of B5. In addition, the coiled-coil structure, or stalk, has been reported to be critical for ligand-induced rupture of the outer EEV membrane (34). The fact that A33-HA interacts with ∆/cc/G/Δ-Strep, a construct that only has the coiled-coil domain of B5 and the transmembrane of VSVG, suggests that the coiled-coil domain of B5 is sufficient for interaction with A33 (Fig. 6). It seems likely that a transmembrane is required by B5 to coordinate its coiled-coil domain for interaction with A33. While the transmembrane domain may not be required for interaction with A33 and EV targeting, we cannot rule out a subsequent function in virion formation or infectivity. Similarly, while these domains are required for EV incorporation, it is still unclear exactly how they function during EV formation. Characterization of recombinant viruses expressing these truncations in place of the normal proteins should help in dissecting their functions.

In our companion paper (4), and elsewhere (31), A33 has been shown to interact with both B5 and B5-GFP during infection. It has also been shown that a recombinant virus expressing B5-GFP makes plaques comparable in size to the parental virus WR, which expresses a normal B5 (41). This would suggest that interaction with A33 is the predominant mechanism for B5 incorporation into EV membranes. B5-GFP was shown to target to the Golgi, the presumed site of intracellular envelopment, when expressed in the absence of other viral proteins (51). Therefore, it is unclear why B5 requires an interaction with both A34 (10, 32) and A33 for incorporation into IEV/EV (5). It is also
unclear why only the lumenal domain of A33 is sufficient for proper targeting. One explanation is that interaction with A33 prevents aberrant interactions by B5 during its progression to the site of wrapping.

The exact role of the transmembrane domain of A33 remains to be determined. Previous work has shown that residues 5 to 40, which constitutes most of the predicted cytoplasmic tail of A33, are not required for its incorporation into IEV (54). The lumenal domain of A33 is sufficient for B5 incorporation into IEV/EV, but A33-HA LD was not detected on B5-GFP labeled VSPs (Fig. 7B). This suggests that the interaction between B5 and A33 is either weak or transient. We can think of two possible explanations for the absence of A33-HA LD on B5-GFP labeled VSPs. The first is that the transmembrane domain of A33 is required for its incorporation into IEV/EVs. This would imply that the A33-B5 interaction is disrupted before IEVs are formed and A33-HA LD is excluded from IEV. Alternatively, the transmembrane domain of A33 is required for retention of A33 in EVs. A33-HA LD should localize to the lumen of the endomembrane system, and therefore the space between the two outer membranes on IEVs. When IEVs fuse with the plasma membrane, this space is now exposed to the media, and unless there is a mechanism to tether A33-HA LD to the CEV, it would diffuse into the media and be lost from the CEV surface. Recombinant viruses expressing A33-HA LD should help determine which scenario is occurring.

A36, and therefore indirectly F12, requires an interaction with A33 for incorporation into IEV membranes (22, 54, 57). B5 is now the second viral protein shown to directly utilize an interaction with A33 for IEV/EV incorporation. The cytoplasmic tail of A33 has been shown to interact with A36 and the interaction is required for the
incorporation of A36 into the wrapping membrane (54, 57). Our results demonstrate that the lumenal domain of A33 interacts with B5. This raises the possibility that these three proteins may exist as a complex. Indeed, mutations in A33 and B5 were found that have the same phenotype in the absence of A36, suggesting a commonality (23, 24). Subsequently, B5 was shown to be involved in an outside-in signaling cascade for actin tail formation via A36 (28). In addition, B5 has been reported to interact with F13 (31). It will be of interest to see if all of these proteins form a complex and if so, how this complex functions in EV formation and determination of the final protein composition of the IEV/EV membranes.

**FIGURE LEGENDS**

Figure 1. B5 is not required for proper localization of A33. HeLa cells grown on glass coverslips were infected with the indicated viruses. The next day, fixed and permeabilized (A) or fixed (B) cells were immunostained with an anti-A33 MAb, followed by Texas Red-conjugated donkey anti-mouse antibody. Localization of B5-GFP or A33 at the site of wrapping (concave arrowhead), at the cell vertices (arrows), and virion-sized particles (arrowhead) are indicated. DNA in the nuclei and viral factories was stained with DAPI or Hoechst (blue). The overlap of B5-GFP (green) and A33 (red) is shown as yellow. Boxed regions are enlarged in the lower left corner to highlight the A33-labeled virion-sized particles.

Figure 2. Incorporation of A33 into EEV does not require B5. (A) Immunoprecipitation. Lysates of purified radiolabeled EEV released from cells infected with the indicated
viruses were immunoprecipitated with an anti-A33 MAb. Protein-antibody complexes were resolved by SDS-PAGE and proteins were detected by autoradiography. Equilibrated EEV lysates were analyzed to verify that equal amounts of EEV were used for immunoprecipitation. The molecular weights, in kDa, and positions of marker proteins are shown. (B) Immunoelectron Microscopy. EEV released from cells infected with the indicated viruses were stained with either anti-B5 MAb or anti-A33 MAb, followed by 18 nm colloidal gold-conjugated goat anti-rat or anti-mouse antibody, respectively. Immunogold-labeled EEV were negative-stained and visualized using a Hitachi 7650 TEM.

Figure 3. A33 and B5 interact through their lumenal domain. (A) Diagrammatic representation of constructs used in co-immunoprecipitation assays. Chimeras were generated from the signal peptide (SP), the coiled-coil domain (cc), the lumenal domain (LD), or the transmembrane domain (TMD) of B5 ( ), VSVG ( ), A33 ( ), or influenza A virus NA ( ). Δ indicates deletion of the corresponding domain and is represented by a dashed line in the diagram connecting included domains. B5 and A33 were tagged with either full-length GFP or an HA epitope, respectively. For B5 and A33, the residue number that starts the predicted domain is shown below the protein diagram. (B) Co-immunoprecipitation. HeLa cells were infected with vTF7.3 in the presence of AraC were transfected with either the indicated plasmids that expressed the constructs described in A or pCDNA3 (Empty Vector). 4.5 h post-transfection, media was replaced with media containing [35S]-Met/Cys. Cells were harvested at 24 h post-transfection,
lysed, and cell lysates were immunoprecipitated with an anti-HA MAb. Immune complexes were analyzed by SDS-PAGE and proteins were detected by autoradiography. (C) Protein expression. Cell lysates used in (B) were subjected to SDS-PAGE and analyzed by Western blot using an anti-GFP (top) or anti-HA (bottom) MAb, followed by an HRP-conjugated donkey anti-mouse or anti-rat antibody, respectively. The molecular weights, in kDa, and positions of marker proteins are shown on the left of blots.

Figure 4. The lumenal domain of A33 is sufficient for interaction with B5-GFP. (A) Diagrammatic representation of constructs used for co-immunoprecipitation. Soluble B5 containing the signal peptide (SP), the lumenal domain (LD) and the coiled-coil domain (cc) of B5 (□). Soluble A33-HA containing the cleavable signal peptide sequence (SP) of VSVG (■) followed by the predicted lumenal domain (LD) of A33 (▲) and the HA epitope tag. Connecting domains that were deleted are represented by a dashed line in the diagram (B) Co-immunoprecipitation. HeLa cells infected with vTF7.3 in the presence of AraC were transfected with either the indicated plasmids that expressed the constructs described in A or pCDNA3 (Empty Vector). 4.5 h post-transfection, the medium was replaced with medium containing [35S]-Met/Cys. Cells were harvested at 24 h post-transfection and lysed. Cell lysates were immunoprecipitated with an anti-HA MAb, immune complexes were resolved by SDS-PAGE, and proteins were detected by autoradiography. (C) Protein expression. Cell lysates used in (B) were resolved by SDS-PAGE and analyzed by Western blot using an anti-B5 (top) or anti-HA (bottom) MAb, followed by an HRP-conjugated donkey anti-rat antibody. The molecular weights, in kDa, and positions of marker proteins are shown on the left of blots.
Figure 5. Membrane anchoring of B5 is required for interaction with A33. (A) Diagrammatic representation of constructs used for co-immunoprecipitation. Chimeric B5-GFP was generated from the predicted luminal domain (LD), the coiled-coil domain (cc) of B5 ( ), and the transmembrane domain (TMD) of VSVG ( ). The A33 construct contains the luminal domain (LD) of A33 ( ), preceded by the cleavable signal peptide sequence (SP) of VSVG ( ). B5 and A33 were tagged with either full-length GFP or an HA epitope, respectively. Δ indicates deletion of the corresponding domain and is represented by a dashed line in the diagram connecting included domains. (B) Co-immunoprecipitation. HeLa cells infected with vTF7.3 in the presence of AraC were transfected with either the indicated plasmids that expressed the constructs described in A or pCDNA3 (Empty Vector). 4.5 h post-transfection, the medium was replaced with medium containing [35S]-Met/Cys. Cells were harvested at 24 h post-transfection and lysed. Cell lysates were immunoprecipitated with an anti-HA MAb, immune complexes were resolved by SDS-PAGE, and proteins were detected by autoradiography. (C) Protein expression. Cell lysates used in (B) were resolved by SDS-PAGE and analyzed by Western blot using an anti-GFP (top) or anti-HA (bottom) MAb, followed by an HRP-conjugated donkey anti-mouse or anti-rat antibody, respectively. The molecular weights, in kDa, and positions of marker proteins are shown on the left of blots.
Figure 6. The coiled-coil domain of B5 is sufficient for interaction with A33. (A) Diagrammatic representation of constructs used in co-immunoprecipitation assays. Chimeras were generated from the signal peptide (SP), the coiled-coil domain (cc), the lumenal domain (LD), or the transmembrane domain (TMD) of B5 ( ), VSVG ( ), A33 ( ). Δ indicates deletion of the corresponding domain and is represented by a dashed line in the diagram. B5 and A33 were tagged with Strep and HA epitope, respectively. For B5 and A33, the residue number that starts the predicted domain is shown below the protein diagram. (B) Co-immunoprecipitation. HeLa cells infected with vTF7.3 in the presence of AraC were transfected with the indicated plasmids that expressed the constructs described in A. Cells were harvested at 24 h post-transfection, lysed, and cell lysates were immunoprecipitated with an anti-HA MAb. Immune complexes were analyzed by Western blot using an anti-Strep MAb (C) Protein expression. Cell lysates used in (B) were subjected to SDS-PAGE and analyzed by Western blot using an anti-Strep MAb followed by an HRP-conjugated donkey anti-mouse (top) or anti-HA (bottom) antisera, followed by a Cy5 conjugated anti rabbit MAb. The molecular weights, in kDa, and positions of marker proteins are shown on the left of blots.

Figure 7. The lumenal domain of A33 is sufficient for interaction with B5-GFP and its incorporation into IEV during infection. (A) Co-immunoprecipitation. HeLa cells were infected with vB5R-GFP/ΔA33R at a MOI of 5.0 and transfected with the indicated plasmids. The next day, cells were harvested by scraping and lysed in RIPA buffer. Cell lysates were subjected to immunoprecipitation with an anti-HA MAb. Immune
complexes (left blots) and cell lysates (right blots) were resolved by SDS-PAGE and analyzed by Western blots using an anti-GFP-HRP and anti-HA-HRP. The molecular weights, in kDa, and positions of marker proteins are shown. (B) *In vivo* trans-complementation analysis. HeLa cells grown on glass coverslips were infected with vB5R-GFP/ΔA33R at a MOI of 1.0 and transfected with the indicated plasmids. The next day, cells were fixed, permeabilized, and immunostained with rabbit anti-HA antibody, followed by Texas Red-conjugated donkey anti-rabbit. Localization of B5-GFP, A33-HA, or A33-HALD at the site of wrapping (concave arrowhead), at the cell vertices (arrows), and virion-sized particles (arrowhead) are indicated. DNA in the nuclei and viral factories was stained with DAPI (blue). The overlap of B5-GFP (green) and A33-HA or A33-HALD (red) is shown as yellow. Boxed regions are enlarged in the lower left corner to highlight the GFP-labeled virion-sized particles.
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envelopment is independent of a large portion of the extracellular domain. J. Virol. 72: 294-302.


Figure 3
B. Interaction

A33-HA
B5-GFP
A33-HA dimers

A33R-HA
B5R

A33R-HA LD

Figure 4
A.

B5-GFP

B5/cc/G/A-GFP

A33R-HA

B.

pA33R-HA

+ + +

Interaction

+ + -

C.

B5-GFP or B5/cc/G/A-GFP

A33R-HA

Figure 5
Figure 6

A.

B5-Strep

B5/cc/B5/Δ-Strep

Δ/cc/B5/Δ-Strep

Δ/cc/G/Δ-Strep

A33-HA

B.

pA33-HA

B5-Strep

B5/cc/B5/Δ-Strep

Δ/cc/B5/Δ-Strep

Δ/cc/G/Δ-Strep

A33-HA

C.

pA33-HA

B5-Strep

B5/cc/B5/Δ-Strep

Δ/cc/B5/Δ-Strep

Δ/cc/G/Δ-Strep

A33-HA
Figure 7