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The Interferon-Induced Gene ISG15 Blocks Retrovirus Release from Cells Late in the Budding Process▽

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The release of retroviruses from cells requires ubiquitination of Gag and recruitment of cellular proteins involved in endosome sorting, including the ESCRT-III proteins and the Vps4 ATPase. In response to infection, cells have evolved an interferon-induced mechanism to block virus replication through expression of the interferon-stimulated gene 15 (ISG15), a dimer homologue of ubiquitin, which interferes with ubiquitin pathways in cells. Previously, it has been reported that ISG15 expression inhibited the E3 ubiquitin ligase, Nedd4, and prevented association of the ESCRT-I protein Tsg101 with human immunodeficiency virus type 1 (HIV-1) Gag. The budding of avian sarcoma leukosis virus and HIV-1 Gag virus-like particles containing L-domain mutations can be rescued by fusion to ESCRT proteins, which cause entry into the budding pathway beyond these early steps. The release of these fusions from cells was susceptible to inhibition by ISG15, indicating that there was a block late in the budding process. We now demonstrate that the Vps4 protein does not associate with the avian sarcoma leukosis virus or the HIV-1 budding complexes when ISG15 is expressed. This is caused by a loss in interaction between Vps4 with its coactivator protein LIP5 needed to promote the formation of the ESCRT-III–Vps4 double-hexamer complex required for membrane scission and virus release. The inability of LIP5 to interact with Vps4 is the probable result of ISG15 conjugation to the ESCRT-III protein, CHMP5, which regulates the availability of LIP5. Thus, there appear to be multiple levels of ISG15-induced inhibition acting at different stages of the virus release process.

One of the important regulatory processes in eukaryotic cells is the ubiquitin-proteasome system, which modulates the fate and function of target proteins through posttranslational attachment of ubiquitin moieties to substrates. Several observations support the view that retroviruses require monoubiquitination signaling for budding (7, 21, 45). For example, PY-late domain-encoding retroviruses, such as avian sarcoma leukosis virus (ASLV), recruit a Nedd4-like E3 ubiquitin ligase for particle release and fragments of the protein are dominant-negative inhibitors of budding (14). Depleting free ubiquitin in cells with proteasome inhibitors blocks the release of human immunodeficiency virus type 1 (HIV-1) and ASLV (26, 27, 39). Covalently linking ubiquitin to the C terminus of equine infectious anemia virus Gag/Δp9 rescues the budding defect caused by the deletion of the YPXL-late domain core motif (13). Although the precise role of ubiquitin in retroviral budding pathways remains unclear, one model posits that ubiquitin promotes the interaction between Gag and host cell factors required for budding. Several of these factors, which form part of the endosomal sorting complex required for transport (ESCRT) pathway, contain ubiquitin-interaction domains that normally bind and sort ubiquitinated cargo proteins during multi vesicular body biogenesis. Approximately 30 proteins directly participate in the ESCRT pathway, organized into high-molecular-weight cytoplasmic complexes, called ESCRT-0 (or HRS-STAM complex), ESCRT-I (Tsg101, Vps28, Vps37, and Mvb12), ESCRT-II (Eap20, Eap30, and Eap45), and ESCRT-III (CHMP1 to CHMP7). Previous findings show that the ubiquitin-binding domain of the ESCRT-I protein, Tsg101 (the UEV domain), binds to both the PTAP motif of HIV-1 Gag and ubiquitin. In fact, ubiquitination of HIV-1 Gag may increase its binding affinity for Tsg101. Forming the HIV-1 budding complex requires the remainder of the ESCRT-I complex and AIP1 but not the ESCRT-II complex (10, 29). In contrast, ASLV Gag requires the Nedd4 E3 ligase and the ESCRT-II (but not ESCRT-I) complex for budding (29, 30). The ESCRT-II complex contains ubiquitin-binding activity through the GLUE domain of Eap45. The last known step in the ESCRT pathway is the oligomerization of the ESCRT-III complex onto membranes to promote membrane scission for vesicle formation and/or virus budding and the subsequent recruitment of the AAA ATPase, Vps4, to catalyze the release of the ESCRT factors from the membrane. This budding mechanism is used by other enveloped viruses including filo-, paramyxo-, and rhabdoviruses (28, 37, 38, 43, 44).

Upon challenge with a foreign pathogen, such as during virus infection, cells begin production of interferons (IFNs), which initiates an innate immune response. IFNs exert autocrine and paracrine actions to upregulate expression of hundreds of IFN responsive genes and induce a cellular antiviral state (34, 36). There are three groups of IFNs (types I, II, and III), classified by the IFN receptor complex they bind. It is the type I IFNs which primarily regulate the antiviral response. Accordingly, recombinant forms of type I IFNs demonstrate therapeutic potential for the treatment of chronic hepatitis B and C virus infections (6). Among the most abundant interferon (IFN)-inducible factors expressed during an antiviral response is the ubiquitin-like protein, ISG15 and its ligase

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complex required to covalently link it to proteins (34, 36). Recent findings have implicated ISG15 as playing an important role in the cellular antiviral response. For example, ISG15 knockout (*ISG15*$^{-/-}$) mice show increased susceptibility to influenza virus, herpesvirus, and Sindbis virus infections, as measured by decreased survival rates compared to wild-type mice (17). Exogenous expression of the conjugation-deficient form of ISG15 fails to protect ISG15$^{-/-}$ mice from Sindbis virus infection (11, 16, 17). In addition, two virus-encoded immune evasion proteins target the ISGylation pathway to enhance replication. The NS1 protein of influenza B virus prevents the interaction of ISG15 with UbE1L to inhibit ISGylation (51). Nairoviruses and arteriviruses encode ovarian tumor domain-containing deubiquitinating enzymes that remove ISG15 from substrates (2, 9).

Treating HIV-1-infected cells with type I IFNs inhibits the release of virus particles (15, 24). This inhibition is dependent on ISG15 expression because a small interfering RNA (siRNA)-mediated knockdown of ISG15 in cells treated with type I IFNs fails to inhibit HIV-1 replication. ISG15 also blocks the interaction between the PTAP late domain motif of HIV-1 Gag and Tsg101, an ESCRT-I protein required for efficient budding of HIV-1 though conjugation of ISG15 was not detected to either HIV-1 Gag or Tsg101 (24). In addition, expression of ISG15 inhibits Ebola virus-like particle (VLP) and HIV-1 VLP release concomitant with decreases in ubiquitination of viral structural proteins (i.e., VP40 or Gag, respectively) (24, 25). The VP40 protein of Ebola virus (EbV) encodes a PY-late domain motif that recruits the E3 ubiquitin ligase, Nedd4, for efficient budding. ISG15 appears to inhibit ubiquitin ligase activity by disrupting the interaction between Nedd4 and the ubiquitin-conjugating E2 enzyme abrogating the transfer of ubiquitin (19, 26). Together, these findings suggest that ISG15 exerts a broad antiviral activity, although the exact mechanism of inhibition appears to be complex.

In this report we show that, like HIV-1 VLPs, ectopic ISG15 expression inhibits release of ASLV VLPs. As with previously described viruses, ISG15 inhibits ubiquitination of ASLV Gag but does not block the interaction between Nedd4 and the PY-late domain motif of ASLV Gag. To ascertain at which point in the retroviral budding pathway is blocked by ISG15, we used previously described Gag-ESCRT chimeras that direct Gag to different stages of the ESCRT pathway. Interestingly, although ISG15 blocked Tsg101 interaction with HIV-1 Gag, providing an ESCRT-I protein in *cis* (HIV P7L-Vps37C chimera) does not bypass the ISG15 mediated inhibition. ISG15 inhibits VLP release and/or ubiquitination of all HIV and ASLV Gag-ESCRT chimeras examined, including ASLV Gag/Δp2b-CHMP6, a poorly budding construct not modified by ubiquitin. This suggests that ISG15 may disrupt particle release late in the budding pathway. To verify this hypothesis, we show that HIV-1 and ASLV Gag associate with Vps4E$_{228}$Q in an L-domain-dependent manner and that ectopic expression of ISG15 blocks this association. The loss of Vps4 from the budding complex is caused by ISG15 conjugation to the ESCRT-III protein, CHMP5, which may regulate the availability of the coactivator protein LIP5 to the ESCRT-III–Vps4 complex required for its activation. In the absence of CHMP5, overexpression of ISG15 no longer prevents LIP5 from binding to Vps4.

MATERIALS AND METHODS

Reagents. All ASV Gag expression constructs were based on plasmid 2036, as previously described (14, 22, 29, 45). The plasmid vector encoding ISG15 was a generous gift from Ronald Harty (University of Pennsylvania) and was previously described (23, 24). The pcDNA3 vectors encoding His$_6$-HA-ISG15, UbE1L, and UbcH8 were kindly provided by Robert M. Krug (52). DNA encoding LDI-1 FL, an avian protein related to Nedd4 (14, 45), and human wild-type Vps4A and Vps4E$_{228}$Q (both a gift from Wesley Sundquist, University of Utah) were previously described (10). HIV-1 Gag-GFP and HIV-1 Gag/P7L-GFP were expressed from the pEGFP-N2 vector, in which green fluorescent protein (GFP) was covalently linked to the C terminus of Gag. The cDNA sequence of CHMP5 was cloned into the pIRES-hrGFP-1a vector (also a generous gift from Wesley Sundquist). A 3×FLAG tag fused to the C terminus of CHMP5 allows for detection of protein with an anti-FLAG monoclonal antibody (Sigma-Aldrich, Milwaukee, WI). The following probes were purchased as indicated: antibodies recognizing: Tsg101, GFP, and HIV CA (p24; Santa Cruz Biotechnology, Santa Cruz, CA); influenza virus HA (Covance, Berkeley, CA); and ISG15 (Cell Signaling Technology, Danvers, MA). The anti-avian myeloblastosis virus MA (which cross-reacts with avian sarcoma virus Gag MA [p19]), developed by David Boettiger, was obtained from the Developmental Studies Hybridoma Bank under the auspices of the National Institute of Child Health and Human Development and maintained by the Department of Biological Sciences at the University of Iowa (Iowa City, IA). Ubiquitinated forms of Gag were detected with a mouse anti-HA serum. Antiserum directed at CHMP5 and LIP5 were a generous gift from Jerry Kaplan and Wes Sundquist (University of Utah).

Transfection of 293/E cells. 293/E cells were cultured in Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum, penicillin (1,000 U/ml), and streptomycin (1,000 μg/ml) to 60% confluence at 37°C. Expression of plasmids from p2036 was high in 293/E cells because these cells stably express the EBNA1 protein of EBV and the p2036 constructs contain the EBV FR plasmid maintenance element that EBNA1 binds. Therefore, 293/E cells were used when proteins expressed from p2036 were to be detected by Western analysis. In all experiments, six-well plates of 293/E cells were transfected with indicated DNA with the FuGENE 6 transfection reagent (Roche Diagnostics, Alameda, CA) according to the manufacturer's instructions. For ubiquitination assay, 293/E cells were transfected with 0.5 to 1.0 μg of the indicated p2036-Gag construct and 0.3 μg of pMT123 encoding HA-tagged ubiquitin.

Detection of proteins by Western blotting. For the budding assay, both media and cell lysate fractions were collected. The cell lysate fractions were prepared by suspension in radioimmunoprecipitation assay (RIPA) buffer (phosphate-buffered saline [PBS] containing 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS], and protease inhibitor mixture tablets) at 48 h posttransfection. VLPs were purified from the cell culture medium by centrifugation through a 20% sucrose cushion at 100,000 × *g* for 1 h at 4°C (Beckman SW50.1 rotor), as previously described (29, 45). The pelleted VLPs were suspended in 100 μl of RIPA buffer containing protease inhibitor mixture tablets. For lysate fractions, Gag proteins were immunoprecipitated overnight at 4°C with a rabbit anti-ASV polyclonal serum or anti-HIV p24 monoclonal serum (1:500 to 1:1,000 dilution) and 20 μl of protein A-agarose beads (45). The precipitated proteins were separated by SDS-PAGE and transferred to a polyvinylidene difluoride membrane. After blocking of the membrane with wash buffer (10 mM Tris-HCl [pH 8.0], 150 mM NaCl, 0.1% Tween 20) containing 5% nonfat dry milk, ASLV Gag proteins were detected with an AMV MA (p19) directed monoclonal antibody (MAb) and an anti-mouse IgG-HRP secondary antibody from ECL (Denville Scientific, Metuchen, NJ). HIV Gag proteins were detected with either anti-HIV CA (p24) MAb or anti-GFP MAb, as indicated. For the CHMP5 ISGylation assay, CHMP5 and CHMP5 conjugates in the lysate was detected after SDS-PAGE using a rabbit anti-CHMP5 serum. Films were reprobed with an anti-HA serum to detect ISG15.

Sedimentation assay. 293/E cells in 6-cm dishes were transfected with the indicated plasmid(s) using FuGENE 6 transfection reagent. At 48 h posttransfection, cells were washed with PBS and solubilized in 350 μl of lysis buffer (10 mM Tris-HCl [pH 7.5], 10% sucrose, 1 mM EDTA, 1% Triton X-100, complete protease inhibitor [Roche Diagnostics]) at 4°C for 60 min. The supernatant and pellet fractions were separated by centrifugation at 10,000 × *g* for 15 min at 4°C. Pellets were suspended into the same volume as the supernatant in lysis buffer and then sonicated for 10 s to shear DNA. Equal volumes of fractions were analyzed by immunoblotting with the indicated antisera.

siRNA depletion of CHMP5 in 293/E cells. siRNA pools directed against the coding sequence of human CHMP5 and a random, nontargeting siRNA sequence were purchased from Dharmacon (Lafayette, CO). The CHMP5 siRNA pool consists of a mixture of four siRNA duplexes; the sense sequences of the

siRNA set are as follows: CAGAAAGCCUUGCGAGUUU, GAAUUUGGA UUGCCACAGA, GAAGGUGUUCCCACUGAUA, and GAGAGGGUCCU GCAAAGAA. Knockdown of CHMP5-FLAG expression was achieved by transfecting 20 to 80 nM CHMP5 siRNA into 293/E cells in six-well plates with RNAiMAX (Invitrogen, Carlsbad, CA). At 48 h posttransfection, the 293/E cells were transfected with plasmid DNA encoding CHMP5-FLAG with the FuGENE 6 transfection reagent as described above. Lysate fractions were harvested 24 h later, and the levels of proteins expression were analyzed by Western blotting as described above.

RESULTS

ISG15 overexpression inhibited ASLV VLP release from 293/E cells. Previous studies demonstrated that overexpression of ISG15 in cells inhibited budding of HIV-1 Gag and Ebola VP40 VLPs (24, 25). In the present study, we sought to determine whether overexpression of ISG15 also inhibited ASLV Gag VLP release. 293/E cells were cotransfected with a plasmid vector encoding wild-type ASLV Gag together with increasing concentrations of a plasmid expressing ISG15. We also included expression plasmids for UBE1L (ISG15 E1 enzyme) and UBCH8 (ISG15 E2 enzyme) required for conjugation of ISG15 to protein. At 48 h posttransfection, Gag was immunoprecipitated from the cell lysate fractions, and VLPs were collected from medium fractions by centrifugation through 20% sucrose cushions. The immunoprecipitated and pelleted proteins were then resolved by SDS–10% PAGE, and Gag proteins detected by Western blotting as described in Materials and Methods. Because proteolytic processing of Gag is not required for particle release, all ASLV Gag constructs contain a D37S mutation in the protease coding region to permit detection of full-length Gag on the gels. As shown in Fig. 1A, expression of ISG15 inhibited ASLV VLP release from 293/E cells, in a dose-dependent manner. In contrast, Gag expression in the lysate fractions was independent of ISG15 expression. β-Actin in the lysate was monitored as a sample loading control. Thus, release of ASLV Gag from cells, like HIV-1, was inhibited by expression of ISG15. If the E1 and E2 enzymes were not transfected into cells, ASLV VLP release was still inhibited but less efficiently (Fig. 1B). These results coincide with previous findings showing overexpression of ISG15 was necessary to inhibit VLP release (24, 25).

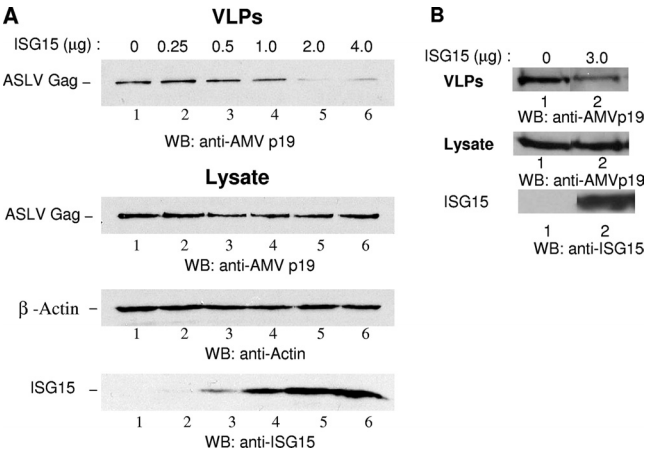

FIG. 1. Expression of ISG15 inhibited ASLV VLP release from cells. (A) 293/E cells were cotransfected with p2036 plasmids (0.5 μg of each) expressing wild-type ASLV Gag, UBE1L, and UBCH8 (lanes 1 to 6) with increasing concentrations of pISG15 (indicated as μg of plasmid DNA transfected into cells. At 48 h posttransfection, Gag was immunoprecipitated from cell extracts. VLPs were collected from the medium fraction by pelleting through a 20% sucrose cushion as described in Materials and Methods. Samples were fractionated by SDS–10% PAGE and immunoblotted with anti-AMV MA (p19) or anti-ISG15 sera. Anti-actin serum was used to detect β-actin as a loading control. (B). 293/E cells were transfected with the p2036 plasmid expressing wild-type Gag (0.5 μg) and the pISG15 plasmid expressing ISG15 (3 μg). Samples were analyzed as in panel A.

ISG15 does not disrupt ASLV Gag-Nedd4 interaction. Retroviral Gag proteins encode late assembly (L) domains that function to recruit components of the ESCRT machinery to the site of budding. The dominant PTAP L-domain motif in the p6 region of HIV-1 Gag binds to the ESCRT-I protein, Tsg101 (10, 46). The PY L-domain motif in the p2b region of ASLV Gag binds to a Nedd4-like E3 protein, which likely links ASLV Gag to the ESCRT-II machinery (14, 22, 29, 45, 48, 49). It was reported that in the presence of ISG15 expression Tsg101 did not bind to HIV-1 Gag (24), and we have confirmed this observation (data not shown). If there is a common mechanism in which ISG15 conjugation interferes with the binding of proteins to their respective L-domain sequences, then the association of the PY motif of ASLV Gag with Nedd4 may also be blocked by the coexpression of ISG15. Therefore, we transfected 293/E cells with the ASLV Gag expression vector in the presence or absence of the ISG15 expression plasmid. As a negative control, cells were transfected with ASLV Gag/Δp2b, an ASLV Gag construct harboring an L-domain deletion. At 48 h posttransfection cell lysate and medium fractions were prepared. In this case, Gag was immunoprecipitated from the lysate fraction with anti-AMV MA (p19) serum. VLPs were isolated from the medium fraction by centrifugation. The proteins from both were subjected to electrophoresis through 10% SDS polyacrylamide gels, and immunoblotting was performed with an anti-Nedd4 serum. Nedd4 precipitates with wild-type ASLV Gag in the presence or absence of ISG15 (Fig. 2, top panel, lanes 1 and 2). Deletion of the L-domain motif abrogated this interaction, verifying that Nedd4 did not precipitate nonspecifically with Gag (Fig. 2, top panel, lane 3). Furthermore, the inhibition of wild-type ASLV Gag particle release in the presence of ISG15 confirmed that the ASLV Gag-Nedd4 interaction still took place under ISG15-induced inhibitory conditions (Fig. 2, bottom panel, lanes 1 and 2). These results suggest that the block to ASLV Gag budding by ISG15 occurred downstream of Nedd4 binding and differed from the result obtained with coexpression of HIV-1 Gag-GFP and ISG15 (24).

ISG15 inhibited budding of HIV-1 Gag/P7L- and ASLV Gag/Δp2b-ESCRT chimeras. If ISG15 inhibition of HIV-1 Gag release resulted from the inability to recruit the ESCRT-I complex, we sought to determine whether covalently linking ESCRT proteins to the C terminus of HIV-1 Gag/P7L could bypass this inhibition. This construct with a point substitution in the PTAP sequence expresses a Gag protein with a budding defect (22). We previously described the capacity of the ESCRT-I protein, Vps37C, and the ESCRT-III protein, CHMP6, to complement the PTAP L-domain mutation when tethered to HIV-1 Gag/P7L (29). Therefore, 293/E cells were cotransfected with plasmid vectors encoding the indicated Gag

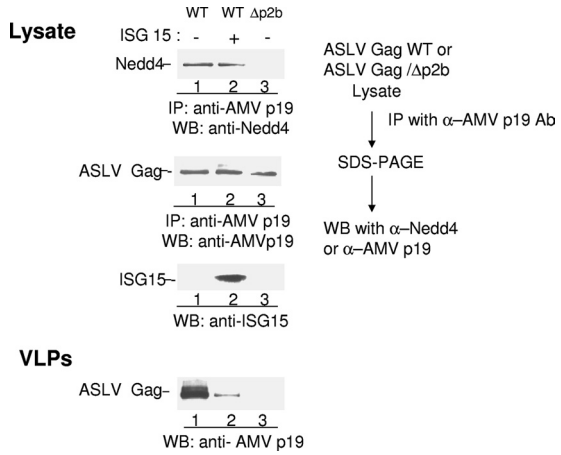

FIG. 2. ISG15 does not block ASLV Gag-Nedd4 association. 293/E cells were transfected with p2036 expressing wild-type ASLV Gag (lanes 1 and 2) or ASLV Gag/Δp2b (lane 3) and, where indicated, with 3 μg of pISG15 (lane 2). At 48 h posttransfection, lysate fractions were immunoprecipitated with anti-AMV MA (p19) serum, and VLPs were collected from the medium fraction as described in the legend to Fig. 1. Samples were resolved by SDS–10% PAGE. Endogenous Nedd4 precipitating with Gag was detected by Western blotting with an anti-Nedd4 serum. Gag in the lysate fraction and VLPs were detected by Western blotting with anti-AMV MA (p19) serum. ISG15 was detected as in legend to Fig. 1.

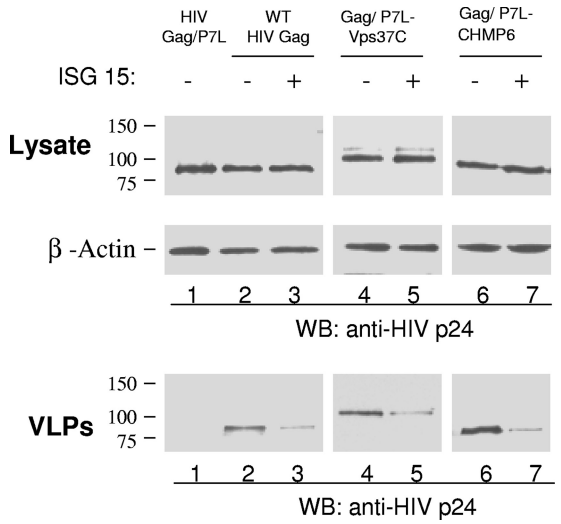

FIG. 3. ISG15 inhibited budding of HIV-1 Gag-GFP and HIV-1 Gag/P7L-ESCRT chimeras. 293/E cells were transfected with p2036 expressing HIV-1 Gag/P7L-GFP (lane 1), Gag-GFP (lanes 2 and 3), Gag/P7L-Vps37C (lanes 4 and 5), or Gag/P7L-CHMP6 (lanes 6 and 7) and, where indicated, with 3 μg of pISG15 (lanes 3, 5, and 7). At 48 h posttransfection, Gag was collected as described in the legend to Fig. 1. Gag expression in lysates and VLP release into the media was detected by Western blotting with anti-HIV-1 CA (p24) serum. All other notations are as in legend to Fig. 1. The positions of migration of molecular weight markers are indicated on the left side of the figures.

constructs in the presence or absence of ISG15 expression. Gag was immunoprecipitated from the cell lysates with an anti-HIV CA (p24) serum, and VLPs were collected from the cell medium fractions by centrifugation through a 20% sucrose cushion. As shown in Fig. 3, ISG15 overexpression inhibited budding of HIV/P7L-Vps37C and HIV/P7L-CHMP6 similar to HIV-1 Gag-GFP.

We next determined if the release of ASLV Gag/Δp2b-ESCRT-I, -II, or -III chimeras, which rescue the L-domain deletion budding defect, were also susceptible to ISG15 inhibition. 293/E cells were transfected with plasmid vectors encoding wild-type ASLV Gag and Gag/Δp2b-ESCRT-I, -II, and -III chimeras in the presence or absence of ISG15. Lysate fractions were immunoprecipitated with an anti-ASLV polyclonal serum, VLPs were collected from the medium fraction, proteins were resolved by SDS–10% PAGE, and Gag was detected by Western blotting with an anti-AMV MA (p19) serum. As shown in Fig. 4, ISG15 overexpression inhibited release Gag/Δp2b-ESCRT-Vps37C, -Eap20, and -CHMP6 chimeras similar to wild-type Gag. The origin of the faster-migrating band observed with the Gag Δp2b-Vps37C fusion is not known but might reflect protease degradation (Fig. 4, lanes 4 and 5). Gag/Δp2b-CHMP6 is not ubiquitinated (28). Therefore, providing ESCRT proteins in *cis* did not alleviate the ISG15 restriction on HIV-1 or ASLV VLP release, suggesting that ISG15 disrupted downstream components of the ESCRT protein-dependent release pathway and in the case of ASLV independent of ubiquitination of Gag.

ISG15 disrupted the association between Vps4E$_{228}$Q and the Gag budding complex. To determine whether ISG15 affects late ESCRT protein assembly, we performed coimmunoprecipitation experiments to examine whether Vps4 was associated with ASLV and HIV-1 Gag budding complexes in the presence of ISG15. Because ESCRT proteins make weak and/or transient interactions with cargo (12a, 35), we performed these experiments with a catalytically inactive form of the enzyme, which has an E$_{228}$Q substitution in its active site (Vps4EQ). This stabilizes interactions within the budding complex. In Fig. 5A, 293/E cells were cotransfected with plasmid vectors encoding wild-type ASLV Gag and hemagglutinin

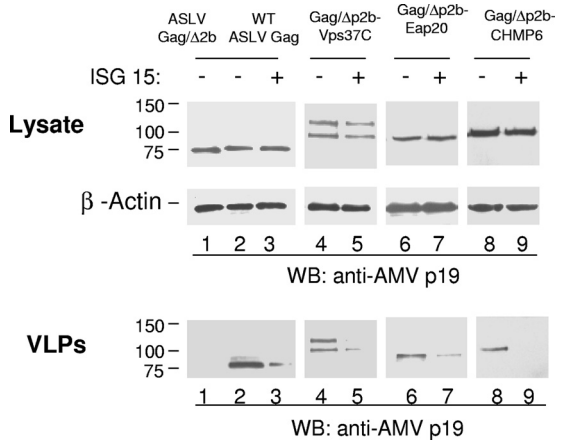

FIG. 4. ISG15 inhibited budding of wild-type ASLV Gag and ASLV Gag/Δp2b-ESCRT chimeras. 293/E cells were transfected with p2036 expressing ASLV Gag/Δp2b (lane 1), wild-type ASLV Gag (lanes 2 and 3), Gag/Δp2b-Vps37C (lanes 4 and 5), Gag/Δp2b-Eap20 (lanes 6 and 7), or Gag/Δp2b-CHMP6 (lanes 8 and 9) and, where indicated, 3 μg of pISG15. At 48 h posttransfection, Gag was collected from lysate and medium fractions and analyzed as described in the legend to Fig. 1. Gag was detected by Western blotting with an anti-MA (p19) serum.

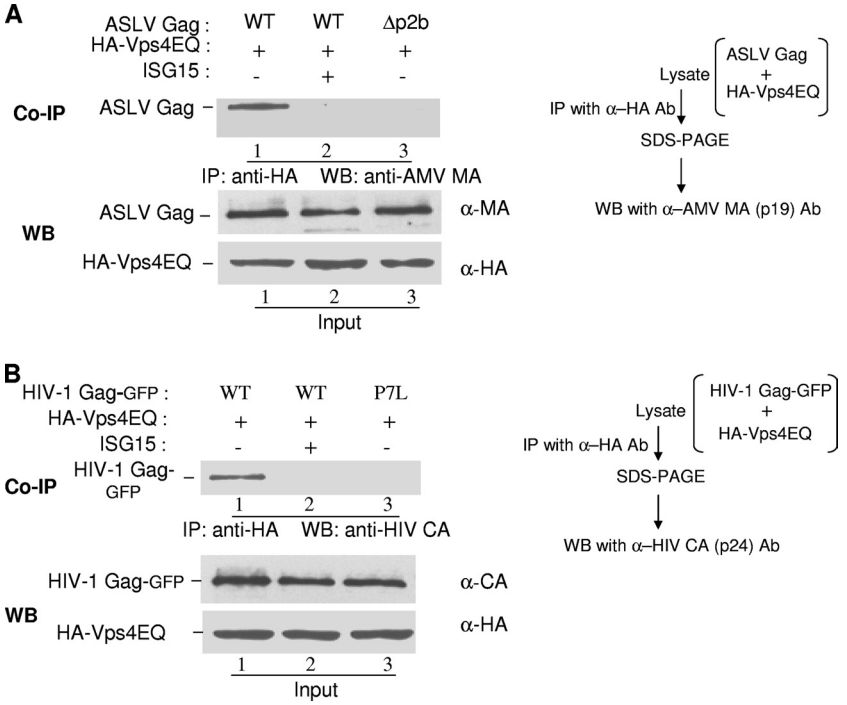

FIG. 5. ASLV and HIV-1 Gag failed to associate with Vps4EQ in the presence of ISG15. (A) 293/E cells were cotransfected with p2036 expressing wild-type ASLV Gag (lanes 1 and 2) or Gag/Δp2b (lane 3) and HA-Vps4E$_{228}$Q (lanes 1 to 3) and, where indicated, pISG15 (lane 2). At 48 h posttransfection, 10% of total cell lysate input was resolved by SDS–10% PAGE to verify the expression of Gag and HA-Vps4EQ by Western blotting with anti-HA or AMV MA serum (bottom panel, marked WB). HA-Vps4EQ was immunoprecipitated with anti-HA antibody from remaining cell lysate fraction and resolved by SDS–10% PAGE. Gag was detected by Western blotting with an antiserum directed at the AMV MA protein (top panel, marked Co-IP). HA-Vps4EQ was detected with the anti-HA serum. (B) 293/E cells were cotransfected with p2036 expressing HIV-1 Gag-GFP (lanes 1 and 2) or HIV-1 Gag/P7L-GFP (lane 3) and HA-Vps4E$_{228}$Q (lanes 1 to 3). An expression plasmid for pISG15 was cotransfected into cells where indicated (lane 2). At 48 h posttransfection, 10% of the total cell lysate input was resolved by SDS–10% PAGE to verify the expression of Gag and HA-Vps4EQ (bottom panel, marked WB). HA-Vps4EQ was immunoprecipitated with anti-HA serum from remaining cell lysate fraction and resolved by SDS–10% PAGE. Gag was detected by Western blotting with anti-HIV-1 CA (p24) serum (top panel marked, Co-IP). HA-Vps4EQ was detected with anti-HA serum.

(HA)-tagged Vps4EQ in the presence or absence of the ISG15-expressing plasmid. At 48 h posttransfection, lysate fractions were immunoprecipitated with an anti-HA serum to pull down HA-Vps4EQ. Immunoblotting with an anti-AMV MA (p19) serum revealed that wild-type ASLV Gag coprecipitated with HA-Vps4EQ (Fig. 5A, upper panel, lane 1). Gag appeared to interact with Vps4EQ in an L-domain-dependent manner because ASLV Gag/Δp2b failed to coimmunoprecipitate with Vps4EQ (Fig. 5A, upper panel, lane 3). In the presence of ISG15 overexpression, wild-type ASLV Gag failed to coprecipitate with HA-Vps4EQ (Fig. 5A, upper panel, lane 2). As a negative control, 293/E cells were cotransfected with plasmid vectors encoding ASLV Gag/Δp2b and an HA-tagged avian homolog of Nedd4, LDI-1 (HA-LDI-1 FL) (14, 45). Pulling down HA-LDI-1 FL with an anti-HA serum failed to coprecipitate Gag/Δp2b, verifying that Gag did not come down nonspecifically with the HA antibody or agarose beads (data not shown). Each of these proteins was expressed in cells independent of ISG15 expression (Fig. 5A, bottom panels).

Similarly, HIV-1 Gag appeared to associate with Vps4EQ in an L-domain-dependent manner. 293/E cells were cotransfected with plasmid vectors encoding HIV-1 Gag-GFP or HIV-1 Gag/P7L-GFP and HA-Vps4EQ (Fig. 5B). Immunoblotting with anti-GFP antibody revealed that wild-type HIV-1 Gag, but not HIV-1 Gag/P7L, bound to Vps4EQ (Fig. 5B, upper panel, lanes 1 and 3). ISG15 expression abolished this interaction (Fig. 5B, upper panel, lane 2). Again, coexpression of each of these proteins with ISG15 was not affected (Fig. 5B, lower panels). Taken together, the results shown in Fig. 5 suggest that ISG15 overexpression prevents the stable recruitment of Vps4 to the site of particle budding.

ISG15 targeted the ESCRT-III complex by ISGylating CHMP5. The loss of Vps4 recruitment and the suggestion that ISG15 inhibited the late stages of budding implicated the ESCRT-III complex as being antagonized by ISG15. The CHMP proteins of the ESCRT-III complex recruit Vps4 to the site of vesicle formation or virus budding upon polymerization on membranes (7, 40). We therefore determined whether ISG15 conjugated to subunits of the ESCRT-III complex. Previously, a proteomics screen of HeLa cells treated with IFN-β identified CHMP5 as a potential substrate for ISG15 modification (52). To confirm this finding, 293/E cells were transfected with plasmid vectors encoding His$_6$-HA-ISG15 (~16 kDa), UBE1L (ISG15 E1 enzyme), and UBCH8 (ISG15 E2 enzyme). At 48 h posttransfection, lysate fractions were analyzed by SDS–10% PAGE. Endogenous CHMP5 (~26 kDa) and its ISG15 conjugate (~42 kDa) were detected by using an anti-CHMP5 serum (Fig. 6, top panel, lanes 2 and 3). The

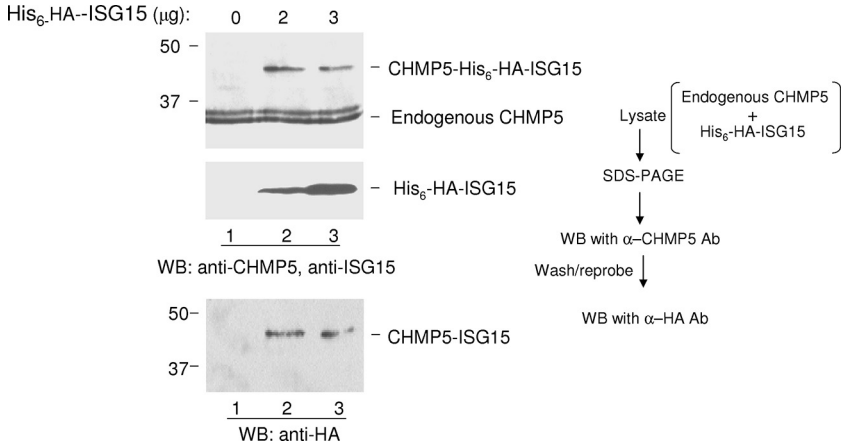

FIG. 6. ISG15 conjugated to the ESCRT-III protein, CHMP5. 293/E cells were transfected with or without plasmids expressing His_6-HA-ISG15, UbE1L, and UbsH8 as indicated. At 48 h posttransfection, cell extracts were resolved by SDS–10% PAGE. (Top panel) Proteins were detected with an anti-CHMP5 serum by Western blotting. The lower and upper bands observed correspond in size to the endogenous CHMP5 and its ISG15 conjugate, respectively. (Middle panel) Total cell lysate (10% of input) was resolved by SDS–10% PAGE to verify expression of His_6-HA-ISG15. (Bottom panel) Blot from panel A was washed and reprobed with an anti-HA serum to detect the His_6-HA-ISG15-conjugated CHMP5.

membrane was reprobed with an anti-HA serum shown in the lower panel. His_6-HA-ISG15 expression is shown in the middle panel. We detected a CHMP5 species migrating at the expected position for a CHMP5-ISG15 conjugate in cells transfected with plasmids expressing His_6-HA-ISG15, UBE1L, and UBCH8 (lanes 2 and 3). In a separate control experiment, we failed to detect a similar band in cells expressing CHMP3-FLAG (32 kDa), an ESCRT-III subunit with a molecular weight similar to that of CHMP5-FLAG (data not shown). These results indicate that a portion of the endogenous CHMP5 is ISGylated.

ISG15 interfered with membrane association of Vps4. Because ESCRT-III proteins cycle on and off membranes, we used a sedimentation assay to detect the subcellular location of both CHMP5 and Vps4 in the presence or absence of ISG15 expression (40). Overexpressed CHMP proteins form detergent-insoluble polymers that capture binding partners on membrane fractions (40). 293/E cells were transfected with a plasmid vector encoding HA-tagged wild-type Vps4 and, where indicated, with CHMP5-FLAG- and ISG15-expressing vectors. At 48 h posttransfection, cells were lysed in a buffer containing 1% Triton X-100 and spun in a centrifuge at 10,000 × *g*. The soluble and the membrane-containing pellet fractions were collected as described in Materials and Methods. Protein samples from both were then resolved by SDS–12% PAGE, and CHMP5-FLAG and HA-Vps4 were detected by Western blotting with an anti-FLAG and anti-HA sera, respectively. When expressed alone, wild-type Vps4 distributed primarily in the soluble, cytosolic fraction (Fig. 7A, lanes 1 and 2), a finding consistent with previous observations. When coexpressed with CHMP5-FLAG, we observed a shift of the Vps4 protein to the pellet fraction with the insoluble CHMP5 aggregates (Fig. 7A, lanes 3 and 4). ISG15 overexpression, however, reversed the tendency of Vps4 to redistribute to the pellet fraction despite the presence of CHMP5 (Fig. 7A, lanes 5 and 6). Strikingly, while the Vps4 was released into the soluble fraction when ISG15 was overexpressed, CHMP5-FLAG accumulated in the membrane pellet fraction (Fig. 7A, bottom panel, lanes 5 to 6). A Western blot to detect β-actin was included as a loading control of samples on the gel.

Because overexpression of exogenous proteins can be associated with experimental artifacts that might be responsible for the changes in multimer status and/or subcellular location, we repeated the above experiments but monitored endogenous Vps4 using an antiserum directed at the protein. As shown in Fig. 7B, endogenous Vps4 is found in the cytoplasm (lanes 1 and 2). In the presence of overexpression of CHMP5-FLAG, Vps4 begins to be recruited to the membrane fraction (lanes 3 and 4). This is reversed when ISG15 is coexpressed in the cells (lanes 5 and 6). We also examined the distribution of endogenous CHMP5 between the soluble and membrane fractions in the presence or absence of ISG15 but without overexpression of Vps4. Endogenous CHMP5 was found mostly in the soluble fraction in the absence of ISG15 expression (Fig. 7C, lanes 1 and 2). In the presence of ISG15, endogenous CHMP5 begins to accumulate in the membrane fraction (Fig. 7C, lanes 3 and 4). The reason for the quantitative difference in the amount of Vps4 and CHMP5 protein that is recruited to membrane fractions (Fig. 7, lanes 3 and 4) is not known but could reflect differences in the concentrations of exogenous versus endogenous proteins. Nevertheless, changes in the distribution of exogenous and endogenous proteins between the soluble and membrane fractions are similar. Taken together, these experiments indicate that ISG15 overexpression caused a significant reduction in Vps4 association with membrane-bound CHMP polymers and an accumulation of CHMP5 on the membrane fraction.

ISG15 inhibited association of the coactivator LIP5 with Vps4. CHMP5 binds to the cellular cofactor, LIP5, which promotes Vps4 assembly from the inactive, cytosolic dimer to the active, membrane-bound double hexamer ring complex (8). We therefore examined whether ISG15 expression disrupted the interaction between LIP5 and Vps4. 293/E cells were cotransfected with plasmids expressing HA-tagged wild-type

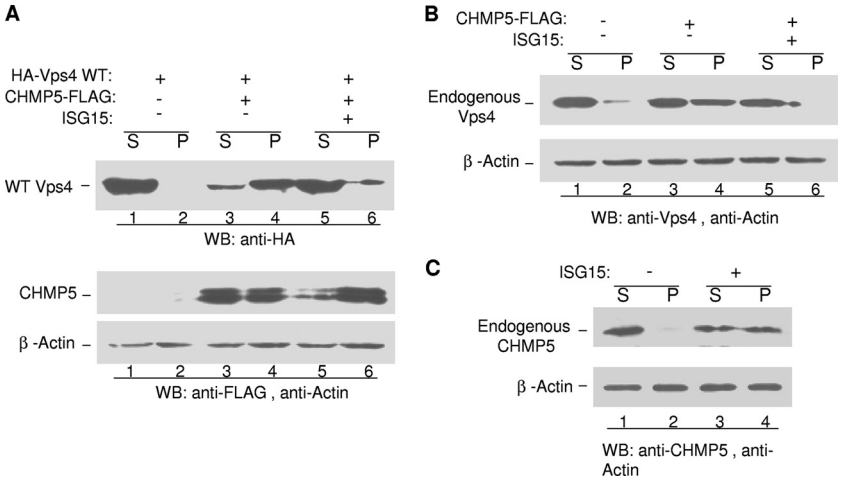

FIG. 7. ISG15 altered membrane association of Vps4 and CHMP5. (A) 293/E cells were transfected with p2036 encoding WT HA-Vps4 and, where indicated, with plasmids expressing CHMP5-FLAG and ISG15. At 48 h posttransfection, cells were lysed, and soluble and pelleted fractions were prepared from the lysate fraction as described in Materials and Methods. Distribution of CHMP5-FLAG and HA-Vps4 WT in the resulting soluble (S) (lanes 1, 3, and 5) and the membrane-bound pellet (P) (lanes 2, 4, and 6) fractions were visualized by Western blotting. (Top panel) Blot probed with anti-HA serum to detect HA-Vps4. (Bottom panel) Blot probed with anti-FLAG serum to detect CHMP5-FLAG expression. β-Actin served as a loading control. (B) 293E cells were transfected with plasmid expressing CHMP5-FLAG and ISG15 where indicated. Cells were lysed and soluble and pellet fractions were prepared as described in panel A. Endogenous Vps4 was detected with an antiserum directed at Vps4. (C) 293E cells were transfected with a plasmid expressing ISG15 where indicated, and samples were prepared as described in panel A. Endogenous CHMP5 was detected with an anti-CHMP5 serum.

Vps4 and FLAG-tagged LIP5. At 48 h posttransfection, cell lysates were prepared and immunoprecipitated with an anti-HA serum, samples were resolved by SDS–10% PAGE, and LIP5 was detected by Western blotting with an anti-FLAG serum. We observed that FLAG-LIP5 coimmunoprecipitated with HA-Vps4 (Fig. 8, lane 1). In the presence of increasing concentrations of ISG15, we detected a dose-dependent decrease in FLAG-LIP5 coprecipitation with HA-Vps4 (Fig. 8, lanes 2 and 3). Western blots of the 10% input of total cell lysates used for coimmunoprecipitation verified consistent expression of HA-Vps4 and FLAG-LIP5. Thus, we conclude that ISG15 reduces Vps4 membrane association by interfering with the LIP5 interaction. Because these experiments were carried out in the absence of virus expression, it implies that ISG15 exerts its effect on Vps4-LIP5 complex at endosomal and other membranes.

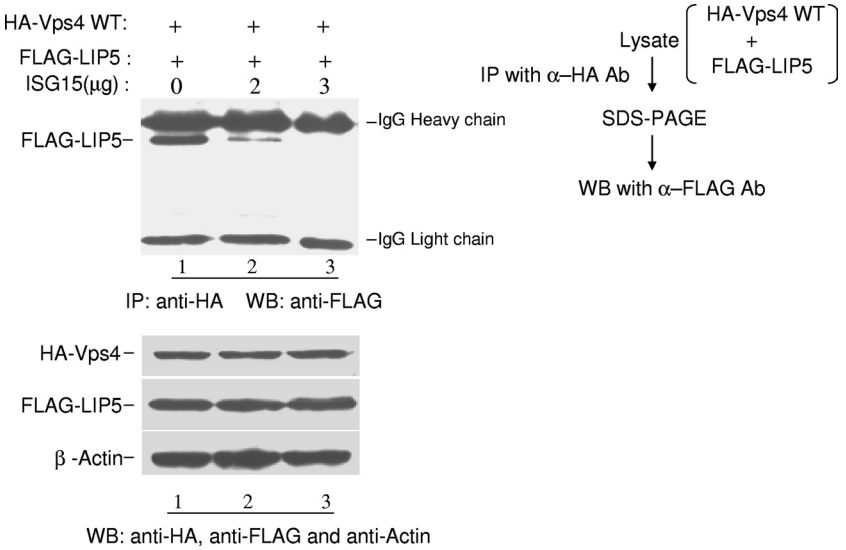

FIG. 8. ISG15 interfered with LIP5-Vps4 interaction. 293/E cells were cotransfected with plasmids expressing HA-Vps4 and FLAG-tagged LIP5 and increasing concentrations of pISG15 where indicated. At 48 h posttransfection, HA-Vps4 was immunoprecipitated from cell extracts with a mouse anti-HA serum, and samples were resolved by SDS–10% PAGE. For the top panel, precipitation of FLAG-LIP5 was determined by Western blotting with anti-FLAG antibody and goat anti-mouse IgG-HRP secondary antibody. The bands above and below FLAG-LIP5 are IgG heavy and light chains, respectively. The bottom panel shows a Western blot of the 10% input of the total cell lysates used in the coimmunoprecipitation assay.

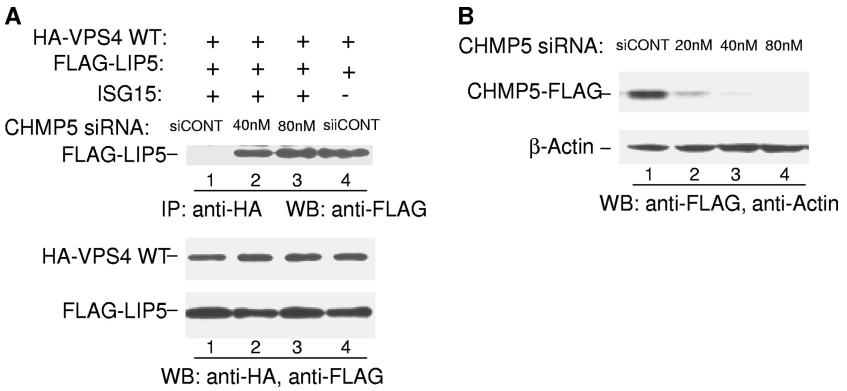

FIG. 9. CHMP5 is required for ISG15-mediated interference of LIP5-Vps4 interaction. (A) 293/E cells were cotransfected with plasmids encoding HA-Vps4, FLAG-LIP5, and ISG15, along with 40 (lane 2) or 80 (lane 3) nM concentrations of a siRNA pool directed against CHMP5 or 80 nM (lanes 1 and 4) of a nontargeting siRNA (siCONT). HA-tagged Vps4 proteins were immunoprecipitated from cell lysate fractions with an anti-HA serum, and precipitated proteins were resolved by SDS–10% PAGE. FLAG-LIP5 was detected by Western blotting with an anti-FLAG antibody. Expression of HA-Vps4 and FLAG-LIP5 were detected as described in the legend to Fig. 8 using anti-HA and FLAG serum. (B) CHMP5-FLAG was expressed in cells in the presence of siCONT (80 nM, lane 1) or the siRNA targeting CHMP5 (20 nM, lane 2; 40 nM, lane 3; 80 nM, lane 4), and proteins were analyzed as in panel A using an anti-FLAG serum. β-Actin again served as a loading control.

CHMP5 was required for ISG15-mediated interference of the LIP5-Vps4 interaction. If ISG15 conjugation to CHMP5 is the key element in controlling the accessibility of Vps4 to its coactivator LIP5, then the removal of CHMP5 would render the budding complex resistant to ISG15 inhibition. To test this hypothesis, we repeated the coimmunoprecipitation experiment described in Fig. 8 but introduced an siRNA directed at CHMP5 or a control siRNA (Fig. 9). As shown in Fig. 9B, the expression of exogenous CHMP5-FLAG was prevented by the specific but not the control siRNA (lanes 1 to 4). β-Actin serves as a gel loading control. When the control siRNA was included (Fig. 9A, lanes 1 and 4), HA-Vps4 coimmunoprecipitated with FLAG-LIP5 was detected in the absence (lane 4) but not in the presence of the overexpression of ISG15 (lane1). In contrast, coimmunoprecipitation of HA-Vps4 with FLAG-LIP5 was restored in the presence of ISG15 when the siRNA directed at CHMP5 was cotransfected into the cells (Fig. 9A, lanes 2 and 3). We conclude from these results that CHMP5 controls the availability of LIP5 to gain access to the Vps4 complex required for oligomerization of the inactive dimer to the active double hexamer.

Because the targeting siRNA but not the control siRNA restored the association of HA-Vps4EQ with LIP5 (Fig. 9), we sought to determine whether the same siRNA targeting CHMP5 would restore the release of ASLV Gag/Δp2b-CHMP6 or HIV-1 GagP7L-CHMP6 VLPs in the presence of ISG15 coexpression. As shown in Fig. 10, lanes 5 to 8, we observed the rescue of both (upper and lower panels, respectively). Similar results were obtained for HIV-1 Gag/P7L-Vps37C, ASLV Gag/Δp2b-Vps37C, and Eap20 (data not shown).

DISCUSSION

Efficient budding of retroviruses depends on the recruitment of the ESCRT machinery through interaction with the L domain. In the late stages of budding, ESCRT-III assembly facilitates membrane scission and recruits Vps4 to the site of particle release for multiple rounds of budding. The ESCRT-III complex consists of ~11 subunits, termed charged multivesicular body proteins (CHMP1 to CHMP7), with characteristic basic N-terminals and acidic C-terminals. Four subunits (CHMP2A/B, CHMP3, CHMP4A/B/C, and CHMP6) form the core of the ESCRT-III complex, with two additional subunits (CHMP1A/B and CHMP5) serving regulatory functions. Recent evidence shows that CHMP1A/B binds to Ist1p, a positive modulator of Vps4 (1, 4, 8, 33). Ist1p contains an MIT-interacting motif (MIM) to bind Vps4 (1, 4). In *Saccharomyces cerevisiae*, Ist1p binding to Vps4 inhibits oligomerization of Vps4 into the catalytically active double hexameric ring structure (8). In both *S. cerevisiae* and mammalian cells, CHMP1 induces Ist1p to endosomal membranes and/or midbodies of dividing cells, which in

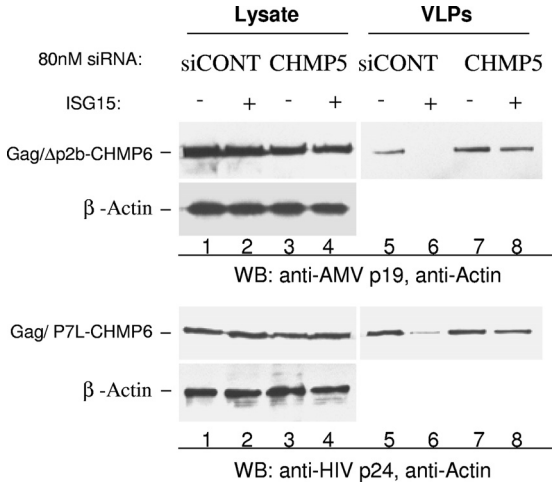

FIG. 10. CHMP5 is required for ISG15-mediated interference of budding by ASLV Gag/Δp2b-CHMP6 or HIV-1 Gag/P7L-CHMP6. 293/E cells were cotransfected with plasmids encoding ASLV Gag/Δp2b-CHMP6 (top panel) or HIV-1 Gag/P7L-CHMP6 (bottom panel) with the ISG15 expression plasmid and control or CHMP5-specific siRNAs (80 nM) as indicated. Lysate and medium fractions were prepared and analyzed as described in legends to Fig. 3 and 4. β-Actin served as a gel loading control.

turn recruits Vps4 to these compartments (4, 8). Paradoxically, overexpression of Ist1p blocked recruitment of catalytically inactive $Vps4E_{233}Q$ to multivesicular bodies presumably by inhibiting oligomerization of Vps4 (8). However, siRNA-mediated depletion of Ist1 does not inhibit HIV-1 Gag budding, suggesting that CHMP1-Ist1 regulation of Vps4 activity is not required for retrovirus release (1, 4). CHMP5, on the other hand, binds to the Vps4 activator, LIP5/Vta1p (40, 47, 50). Unlike Ist1, LIP5 promotes oligomerization of Vps4, thus stimulating ATP hydrolysis (3, 4). In fact, Ist1p competes with LIP5/Vta1p for Vps4 binding in *S. cerevisiae* (8). Suppression of LIP5 expression inhibits HIV-1 Gag budding, revealing a role for LIP5-mediated regulation of Vps4 in retrovirus budding (47).

ISG15 has been implicated in an interferon induced mechanism for inhibition of virus release from cells. It is expressed as a 165-amino-acid precursor, which contains two tandem domains with significant homology to ubiquitin (5). Like the ubiquitination reaction, ISGylation requires a cascade of enzymes to conjugate ISG15 to substrates (6, 31, 34), and these enzymes are also induced by interferon. Despite the similarities to ubiquitin, ISG15 does not promote degradation of substrates and may even target a distinct population of proteins (18). In the present study, we now show that ISG15 exhibited antiviral activity against ASLV VLP release, similar to its effects on HIV-1 VLPs, even though the two viruses use parallel pathways to release from cells (22, 29). One mechanism proposed for inhibition of virus release by ISG15 is that it prevents ubiquitination of viral structural proteins, such as Gag (19, 25). As with HIV-1 Gag, ISG15 overexpression impedes ubiquitin modification of wild-type ASLV Gag or Gag/Δp2b-ESCRT-I and -ESCRT-II chimeras (data not shown). Because the Nedd4-ASLV Gag interaction is not interrupted by ISG15, this finding is consistent with the inhibition of the ubiquitin ligase activity of Nedd4 by ISG15 as a mechanism for inhibition for ASLV release. This suggests that ISG15 broadly disrupts the ubiquitination reaction, possibly by suppressing the activity of ubiquitin-conjugating E2 enzymes (42, 53). However, the precise mechanism of how the loss of ubiquitination leads to a budding defect remains unclear. Reconstitution of a Nedd4-independent budding pathway by covalently linking ESCRT proteins to the C terminus of ASLV Gag/Δp2b did not negate ISG15 inhibition. ISG15 also inhibited budding of an ASLV Gag/Δp2b-ESCRT-III chimera (Gag/Δp2b-CHMP6) that is not modified by ubiquitin (Fig. 4, lane 8). In a similar fashion, covalently linking the ESCRT-I protein, Vps37C, or the ESCRT-III protein, CHMP6, to the C terminus of HIV-1 Gag/P7L did not bypass ISG15 inhibition either. Because these chimeras cause ASLV and HIV-1 Gag to enter their respective budding pathways at late steps in the process, these results indicate that ISG15 may inhibit budding through additional mechanisms other than blocking ubiquitin modification of Gag.

We and others have demonstrated the inability of HIV-1 Gag to bind Tsg101 in the presence of ISG15. Okumura et al. (24) hypothesized that the loss of ubiquitin modification decreases the affinity between HIV-1 Gag and Tsg101 and destabilizes the budding complex. However, the binding affinity of the UEV domain of Tsg101 for ubiquitin is very weak relative to its affinity for the PTAP L-domain motif. In fact, mutations in the UEV domain that disrupt ubiquitin binding only marginally decreased PTAP binding (20, 32, 41). Mutating ubiquitin-acceptor sites in the p6 region of HIV-1 Gag did not inhibit budding, implying that the Tsg101-Gag interaction remained unencumbered (12).

We now demonstrate that ISG15-mediated inhibition of ASLV and HIV-1 budding was caused by the failure of Vps4 to associate with the retrovirus budding complex in the presence of the overexpression of ISG15 (Fig. 6). The loss in budding was associated with a significant decrease in Vps4 cosedimentation with insoluble CHMP5 polymers on cell membranes (Fig. 7). Vps4 catalyzed ATP hydrolysis provides the energy for disassembly of the membrane-bound ESCRT complexes during the release process. Thus, the inability to recruit Vps4 to the budding complex may cause ESCRT complexes to remain trapped on membranes unable to cycle off into the cytoplasm. This may explain the accumulation of CHMP5 on membrane fractions, as well as the loss of HIV-1 Gag binding to Tsg101 in the presence of ISG15. The probable mechanism by which ISG15 inhibits Vps4 binding to membrane-bound ESCRT-III complex is that it targets the Vps4-LIP5 complex (Fig. 8) through the ISGylation of CHMP5 (Fig. 6). When CHMP5 expression is suppressed in cells, the association of Vps4 with its coactivator, LIP5 (Fig. 9), is restored. These results also have broad implications for the cellular MVB process, in which ISG15 will likely inhibit membrane protein sorting. In preliminary experiments using an Env-deficient HIV-1 proviral luciferase reporter construct and VSV-G, the release of virus was blocked by ISG15 expression. This was partially reversed in the presence of the siRNA pool targeting CHMP5 but not the control siRNA.

A model for ISG15 inhibition of virus release applicable to retroviruses, as well as other enveloped viruses, is presented in Fig. 11. During assembly, the ESCRT-III complex polymerizes around the membrane neck of a budding retrovirus particle. MIM domain-containing proteins recruit the inactive Vps4 dimer to the ESCRT-III complex. In the presence of both ATP and LIP5, Vps4 oligomerizes into an active double-hexameric ring structure that disassembles the ESCRT complex and facilitates multiple rounds of budding (Fig. 11A). In the presence of ISG15, however, Vps4 fails to bind LIP5, and the active oligomeric enzyme does not form. As a result, Vps4 remains primarily cytosolic, ESCRT complexes remain trapped on membranes, and virus budding is blocked (Fig. 11B). Although LIP5 also binds to the MIM domains of other ESCRT-III proteins, CHMP5 lacks this domain. CHMP5 may regulate the interaction between LIP5 and Vps4 by preventing LIP5 from prematurely binding MIM domain-containing proteins in the ESCRT-III complex. Accordingly, siRNA depletion of CHMP5 increased the release of infectious HIV-1 particles, suggesting that CHMP5 may negatively regulate LIP5-Vps4 (47). Conjugation of ISG15 to CHMP5 may sequester LIP5, although this remains to be determined. Removal of CHMP5 prevents ISG15 from inhibiting the association of the Vps4 ATPase with its cofactor LIP5.

Thus, there appears to be at least two levels of ISG15-mediated inhibition of virus release acting at different stages of budding. In an early stage of the release process, ISG15 broadly disrupts ubiquitin signaling, which inhibits ubiquitin modification of Gag, as well as host cell factors required for budding (i.e., Tsg101 [24]). In addition, ISG15 acts late in the

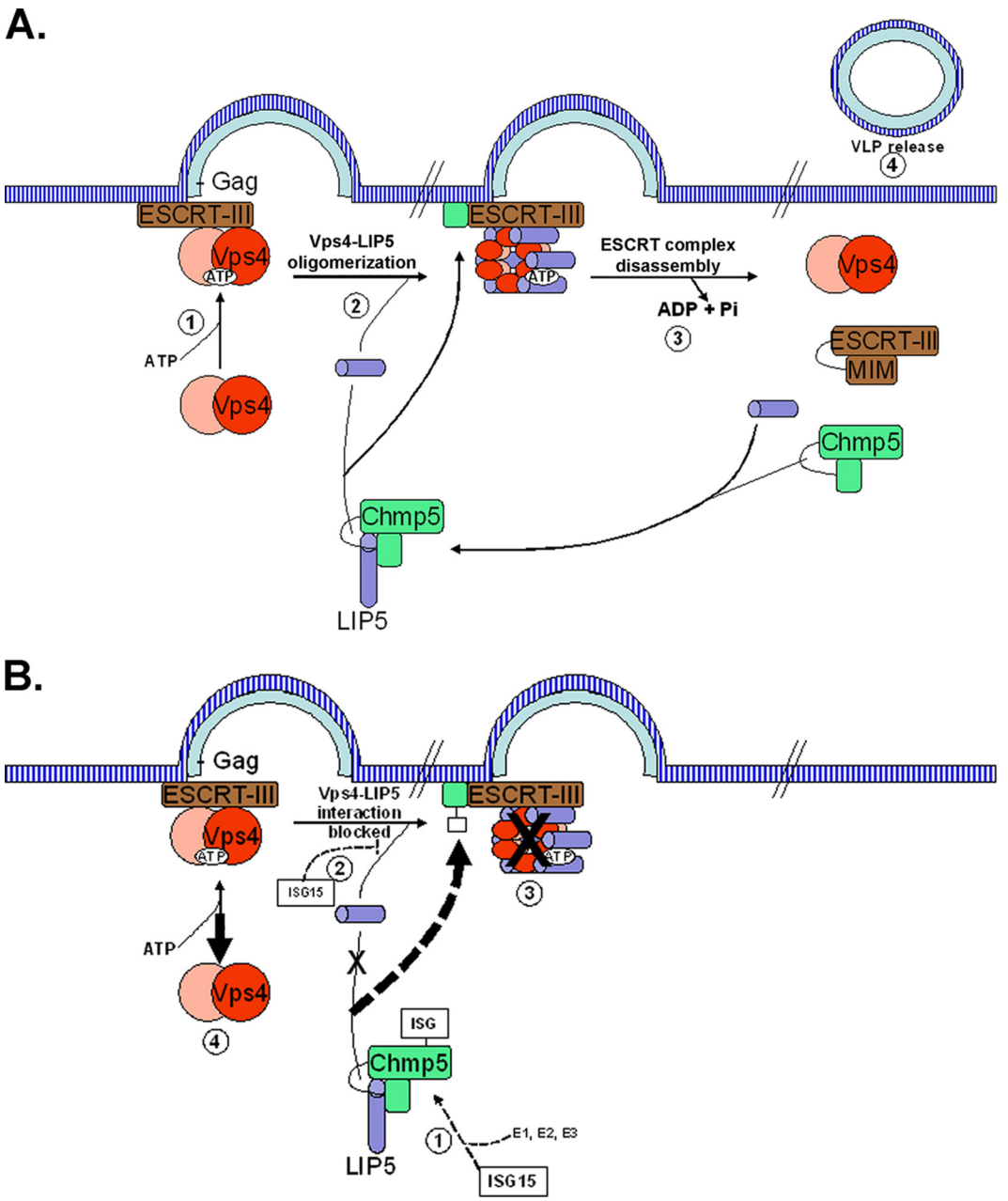

FIG. 11. Model of the role of Vps4 in retrovirus budding. (A) Vps4 activity is regulated by the oligomeric state of the enzyme. In its inactive form, Vps4 exists as a dimer in the cytosol. (Step 1) Upon polymerization of the ESCRT-III complex with Gag, the ATP-bound Vps4 dimer is recruited to membranes through its interaction with MIM domains found in most ESCRT-III proteins. (Step 2) ATP-bound Vps4 dimers coassemble into double hexameric ring complexes with the coactivator protein, LIP5, to form the active enzyme. LIP5 binds the ESCRT-III protein, CHMP5, in the cytosol. Although CHMP5 can localize to membranes, the literature is unclear whether the LIP5-CHMP5 interaction persists once LIP5 coassembles with Vps4. (Step 3) ATP hydrolysis by the Vps4-LIP5 oligomer releases the ESCRT complexes from membranes and returns Vps4 to its inactive dimeric form in the cytoplasm. (Step 4) Dissociation of the ESCRT complex coincides with the membrane fission event to release retrovirus particles from the cell surface. (B) ISG15 prevents Vps4-LIP5 oligomerization. (Step1) The ESCRT-III protein, CHMP5, binds to LIP5 in the cytosol possibly preventing it from binding to MIM domains of the ESCRT-III complex. In the presence of ISG15-specific E1 and E2 enzymes, ISG15 conjugates to CHMP5. (Step 2) ISG15 overexpression blocks the interaction between Vps4 and its coactivator, LIP5. Although the function of CHMP5 remains largely uncharacterized, ISGylation of CHMP5 may alter Vps4-LIP5 complex assembly by sequestering LIP5. (Step 3) In the absence of the Vps4-LIP5 oligomer, ESCRT complexes remain bound to membranes and virus budding is arrested. (Step 4) Vps4 is released into the cytosol.

release process by negatively regulating Vps4 ATPase activity by targeting the CHMP5-LIP5 complex. Since siRNA-mediated knockdown of CHMP5 expression rescues HIV-1 virus release in the presence of ISG15, this indicates that the latter mechanism may be the primary mechanism of inhibition. The finding that an ISG15-dependent mechanism targets the last step in budding takes on added importance because it represents biological selection of a general host antiviral mechanism

directed at many enveloped viruses. These findings also explain why there is strong counter selection for viral enzymes that prevent ISGylation of proteins (2, 9).

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