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Article

HIV-1 Antagonism of CD317 Is Species Specific and Involves Vpu-Mediated Proteasomal Degradation of the Restriction Factor

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SUMMARY

Mammals encode proteins that inhibit viral replication at the cellular level. In turn, certain viruses have evolved genes that can functionally counteract these intrinsic restrictions. Human CD317 (BST-2/HM1.24/tetherin) is a restriction factor that blocks release of human immunodeficiency virus type 1 (HIV-1) from the cell surface and can be overcome by HIV-1 Vpu. Here, we show that mouse and rat CD317 potently inhibit HIV-1 release but are resistant to Vpu. Interspecies chimeras reveal that the rodent-specific resistance and human-specific sensitivity to Vpu antagonism involve all three major structural domains of CD317. To promote virus release, Vpu depletes cellular pools of human CD317, but not of the rodent orthologs, by accelerating its degradation via the 20S proteasome. Thus, HIV-1 Vpu suppresses the expression of the CD317 antiviral factor in human cells, and the species-specific resistance to this suppression may guide the development of small animal models of HIV infection.

INTRODUCTION

Besides classical innate and acquired immune responses, mammals have evolved a set of dominant genes that are capable of suppressing or preventing virus replication at the host cell level. Among these major cellular defenses against retroviral infection are the Friend virus susceptibility-1 (Fv1) gene product and members of the tripartite interaction motif (TRIM) family, both of which target incoming retroviral particles (Goff, 2004; Sebastian and Luban, 2007; Towers, 2007), with some TRIM proteins apparently also interfering with transcription (Wolf and Goff, 2007) or late steps in the retroviral replication cycle (Sakuma et al., 2007; Uchil et al., 2008). A second major cellular antiviral activity has been identified for members of the apolipoprotein editing complex (APOBEC) class of cytidine deaminases that hypermutate and/or destabilize viral genomes, including HIV, Moloney murine leukemia virus, hepatitis B virus, human papilloma viruses (Vartanian et al., 2008), and endogenous retroelements (Chiu and Greene, 2008). These restriction factors, which typically are constitutively expressed but can frequently be upregulated by host cells in response to virus infection, appear to impose particularly effective barriers in the context of cross-species transmission of viruses (Chiu and Greene, 2008; Towers, 2007). Accordingly, they have been classified as belonging to the "intrinsic" immunity, representing an innate cellular network for the front-line defense in an immunologically naive host (Bieniasz, 2004).

Recently, human CD317 was identified as an additional IFNα-inducible restriction factor, the expression of which prevents the final step in the release of fully matured HIV particles from infected cells through surface-protein-based tethers (Neil et al., 2008; Van Damme et al., 2008). Accordingly, the molecule has also been referred to as "tetherin" (Neil et al., 2008). In addition, human CD317-expressing cells apparently display an enhanced endocytosis of mature virions from the surface into CD63-positive compartments. The physiological function of CD317, however, is currently unknown. CD317 is an integral membrane protein expressed constitutively on terminally differentiated B cells (Goto et al., 1994), bone marrow stromal cells (Ishikawa et al., 1995), and plasmacytoid dendritic cells (Blasius et al., 2006). It has been noted that CD317 can be upregulated in most cell types following IFN stimulation (Blasius et al., 2006).

HIV-1 encodes a unique set of accessory gene products to optimize its replication in the human host, and this is achieved in part by counteracting host restriction factors (Malim and Emerman, 2008; Simon et al., 1998; Varthakavi et al., 2003). The viral Vif protein, for example, depletes cellular APOBEC3G pools and thus prevents APOBEC3G encapsidation into HIV particles, ensuring high infectivity of viral progeny (Chiu and Greene, 2008). The HIV-1 accessory protein Vpu and several HIV envelope glycoproteins share the ability to overcome a dominant restriction of virion release in certain human cells, including T cells and macrophages (Bour et al., 1996; Göttlinger et al., 1993; Iida et al., 1999; Klimkait et al., 1990; Noble et al., 2006; Ritter et al., 1996; Schubert et al., 1999, 1995; Strebel, 2007; Varthakavi et al., 2003). The observation that Vpu can counteract CD317-mediated release inhibition (Neil et al., 2008; Van Damme et al., 2008) suggested that CD317 may constitute this long-sought restriction factor. The mechanism by which Vpu counteracts

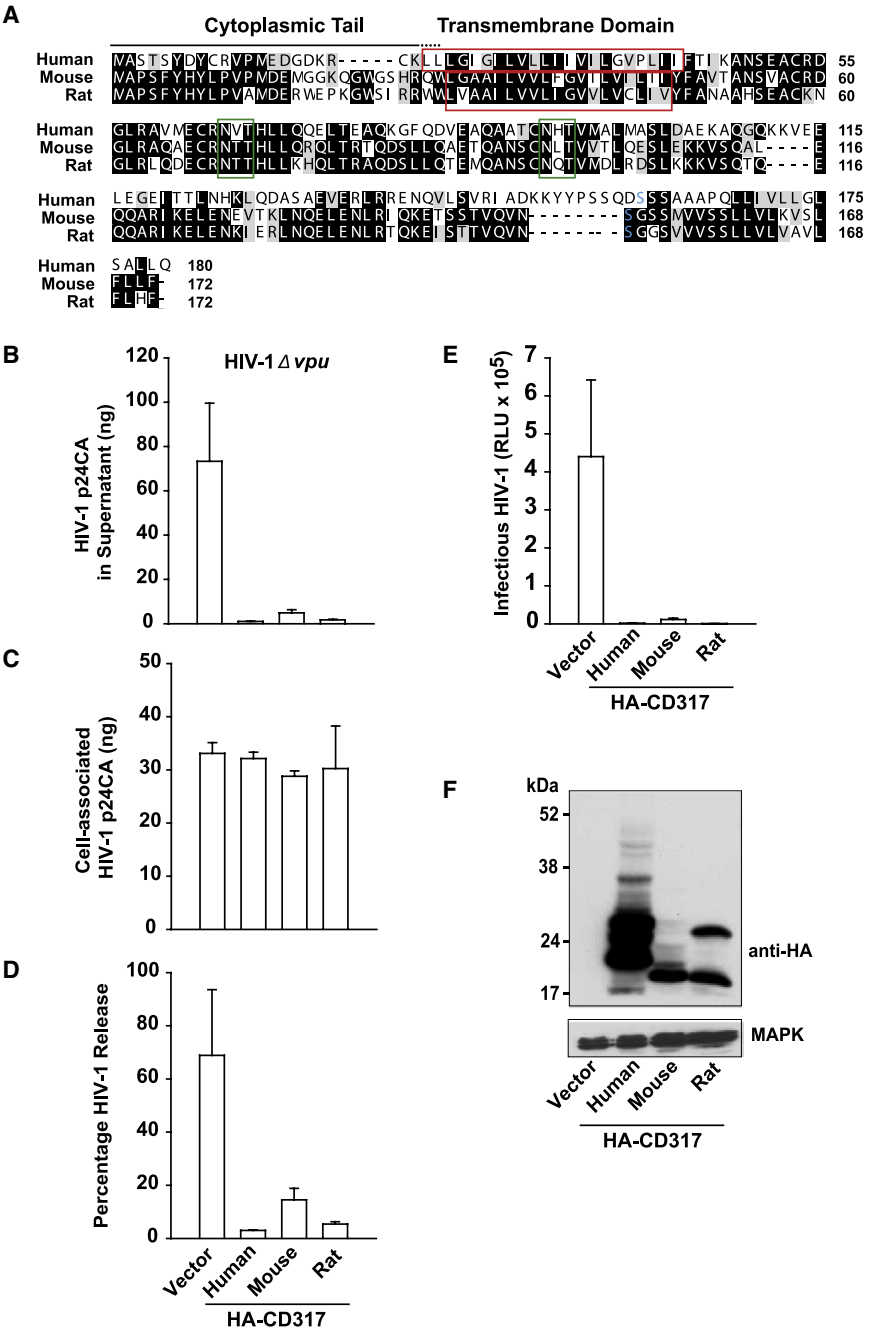

Figure 1. Human, Mouse, and Rat CD317 Restrict HIV-1 Particle Release

(A) Sequence alignment of CD317 of human, mouse, and rat origin (ClustalW2 method and BOXSHADE). Identical amino acids are shaded in black, conserved or similar residues are in gray, and unrelated amino acids in white (identity scores determined by Vector NTI). The predicted, unusual topology (Kupzig et al., 2003) includes an amino-terminal cytoplasmic tail; sequences boxed in red indicate the predicted position of the transmembrane domain. In the extracellular coiled-coil domain, the two putative N-glycosylation sites are boxed in green, and the serine residues that are predicted as the site of cleavage prior to C-terminal addition of a GPI anchor is highlighted in blue.

(B–E) Impact of HA-tagged CD317 from all three species in HIV-1Δ*vpu*-expressing 293T cells on the yield of virion-associated p24CA antigen (B) and infectious HIV-1 virions in culture supernatants (E) and levels of cell-associated p24CA (C). Effect of CD317 on HIV-1 release was expressed as the percentage of total p24CA (in cells and supernatant) that was secreted as virion-associated p24CA in (D). Values are arithmetic means ± SD of triplicates from one representative experiment out of four.

(F) Corresponding western blot analysis of 293T cell lysates for HA-CD317 and MAPK expression.

human CD317 as well as host species-specific differences in the activity of the intrinsic immunity factor (Neil et al., 2007) are therefore particularly important topics.

In the current study, we report that the release-specific antiretroviral activity of CD317 is conserved between the human and rodent orthologs in spite of extensive amino acid sequence divergence. Analysis of the activity of the virus-encoded antagonist Vpu unmasks a striking difference for the interaction of the human pathogen with the three CD317 orthologs. Mechanistically, cellular levels of human but not of rodent CD317 are reduced in the presence of Vpu, resulting in a rescue of HIV-1 release. Vpu achieves this depletion by targeting human CD317 for degradation via the 20S proteasome. Our results highlight CD317-mediated antiretroviral defense and viral evasion of this intrinsic immune factor as remarkable examples of the constantly evolving battle of retroviruses with natural and potential hosts.

RESULTS

CD317 from Human, Mouse, and Rat Inhibits HIV-1 Release

The amino acid sequences of mouse (m) and rat (r) CD317 display extensive divergence from their human (h) ortholog, sharing only 41% and 36% sequence identity, respectively (Figure 1A). The rodent orthologs are 71% identical to each other. We first tested whether anti-HIV-1 activity is a conserved function of CD317 in spite of this sequence diversity. To assess their effect on HIV-1 production, N-terminally HA-epitope-tagged CD317 proteins were coexpressed with Vpu-defective HIV-1 (HIV-1Δ*vpu*) in human 293T cells, which normally harbor low or no endogenous hCD317 expression (Neil et al., 2008; Van Damme et al., 2008). The three CD317 proteins displayed species-specific migration patterns on SDS-PAGE (Figure 1F), reflecting differential N-glycosylation (Figure S1) (Kupzig et al., 2003). The yield of Vpu-defective HIV-1 virions was drastically reduced relative to the vector control following transient expression of all three CD317 orthologs, as assessed

by levels of HIV-1 p24CA antigen (15- to 73-fold reduction) (Figure 1B) and infectious HIV-1 (39- to 650-fold reduction) (Figure 1E) that were released into culture supernatants. In contrast, cell-associated levels of p24CA were nearly identical in the presence or absence of CD317 (Figure 1C). Based on the values shown in Figures 1B and 1C, the effect of CD317 on HIV-1 release was expressed as the percentage of total p24CA (in cells and supernatant) that was secreted as virion-associated p24CA (Figure 1D). This approach has been used previously by others (Bour et al., 1999; Van Damme et al., 2008; Varthakavi et al., 2008). Thus, as observed for hCD317, CD317 from mouse and rat potently restricts the production of Vpu-defective HIV-1.

Rodent and Human CD317 Restrict the Release of Mature HIV-1 Virions

To address whether CD317 proteins have an impact on the synthesis, processing, or degradation of HIV-1 Gag, [^{35}S]methionine pulse-chase radiolabeling studies were performed in 293T cells cotransfected with an expression plasmid encoding codon-optimized HIV-1 GagPol and expression plasmids for the different HA-CD317 proteins or HA alone (vector). A concurrent virological assessment under these experimental conditions showed a strong inhibition of release of Env-deficient HIV-1 virus-like particles (Figure S2), similar to that observed with Vpu-defective HIV-1 (Figures 1B–1D). After 30 min pulse-labeling, amounts of the unprocessed Pr55Gag precursor and processed p24CA were comparable or slightly reduced by CD317 coexpression, and, expectedly, no released particles could be detected (Figure 2A, left panels). After a 4 hr chase period, the majority of Pr55Gag was processed to p24CA under all conditions (Figure 2A, right panels). Most significantly, the release of radiolabeled virions was markedly reduced upon coexpression of CD317 from all three species, consistent with the steady-state analyses in Figure 1B. Thus, neither human nor rodent CD317 have a pronounced effect on HIV-1 Gag synthesis and processing, but impair the release of newly assembled, mature virions.

We next assessed whether expression of rodent CD317 induces an accumulation of HIV-1 Gag and virions at the cell surface and in vesicular compartments as reported for the human ortholog, applying a morphological quantification scheme introduced by Neil and colleagues (Neil et al., 2008). The anti-p24CA staining of HIV-1Δ*vpu*-transfected 293T cells showed a diffuse punctate distribution in the cytoplasm (Figure 2B, vector), while expression of all three HA-tagged CD317 proteins induced a marked redistribution of the major structural Gag protein, highlighted by its enrichment in large aggregates at the plasma membrane and/or intracellularly (Figures 2B and S3 for quantification). In these HIV-1-expressing cells, HA-CD317 proteins predominantly localized to intracellular compartments/large vesicular structures frequently found in a ring-like pattern underneath the plasma membrane or around the nucleus; this is consistent with the previously described localization of intracellular CD317 (Kupzig et al., 2003). It is of note that provirally encoded HIV-1 Gag and HA-CD317 typically did not colocalize, which is in contrast to results reported for a codon-optimized Gag.GFP fusion protein (Neil et al., 2008).

Immunoelectron microscopy of cells cotransfected with plasmids expressing HIV-1_{NL4-3} wild-type (HIV-1 WT) and HA-CD317 revealed clustered virions at or underneath the plasma membrane, which were strongly labeled with anti-p24CA (Figure 2C [15 nm gold, arrowheads]). HA-hCD317 was frequently found in vesicular structures adjacent to viral particles (Figure 2C [10 nm gold, arrow]) with weaker staining of the plasma membrane. HIV-1 budding sites and virus particles exhibited no apparent enrichment in anti-HA labeling compared to the surrounding plasma membrane (Figure 2C and data not shown).

Thin-section electron microscopic analysis of epoxy resin sections from 293T cells transfected with HIV-1Δ*vpu* provirus and a control vector showed occasional immature and mature virions associated with the plasma membrane (Figure 2D, vector). In contrast, expression of CD317 from humans or rodents resulted in accumulation of mature virions attached to the surface or to each other in a fair number of cells (Figure 2D). Mature virions were also observed in what appear to be intracellular compartments in CD317-expressing 293T cells (Figure 2D [arrows]); however, the identity of these compartments and their possible communication with the cell surface is currently unclear.

Together, these results demonstrate that human and rodent CD317 do not markedly interfere with HIV-1 Gag synthesis and processing, but share the ability to inhibit release of infectious HIV-1 by leading to the tethering of mature particles to the surface of virus-producing cells and by inducing the accumulation of virions in plasma membrane-adjacent compartments.

The Rodent Orthologs of CD317 Are Resistant to Counteraction by HIV-1 Vpu

Recent studies demonstrated that the inhibition of virion release by CD317 from humans can be antagonized by Vpu (Neil et al., 2008; Van Damme et al., 2008). Here, we explored the susceptibility of the HIV-1 restriction imposed by mouse and rat CD317 to Vpu antagonism. First, virion production was quantified from 293T cells cotransfected with decreasing amounts of expression plasmids encoding authentic, untagged CD317 from all three species and a proviral plasmid encoding either HIV-1 WT or the isogenic Vpu-defective counterpart, HIV-1Δ*vpu*. In the absence of ectopic CD317 expression, levels of virions in the supernatant were in a similar range for HIV-1 WT and HIV-1Δ*vpu* (Figure 3A, vector), confirming recent reports (Neil et al., 2008; Van Damme et al., 2008). The highest levels of ectopically expressed hCD317 potently reduced HIV-1 release and induced virion tethering irrespective of the Vpu status (Figures 3A and 3B; see also EM images for HIV-1 WT in Figure S4). Titration of the human restriction factor, however, revealed that at lower levels of hCD317 expression, particle release was rescued for HIV-1 WT, but not for Vpu-defective HIV-1 (Figures 3A and 3B, left panels). In stark contrast, the ability of m- and rCD317 to inhibit particle release was virtually indistinguishable for HIV-1 WT and HIV-1Δ*vpu* (Figures 3A and 3B, middle and right panel). Collectively, provirally encoded Vpu antagonizes the anti-HIV-1 activity of hCD317, but this counteraction can be overcome at high levels of the restriction factor. The impact of the rodent orthologs on HIV-1 release, on the other hand, is Vpu-resistant.

To corroborate and extend these findings, we used an experimental setup that allowed us to assess the effect of different expression levels of the viral antagonist Vpu on CD317-mediated inhibition of HIV-1 release. 293T cells were cotransfected with

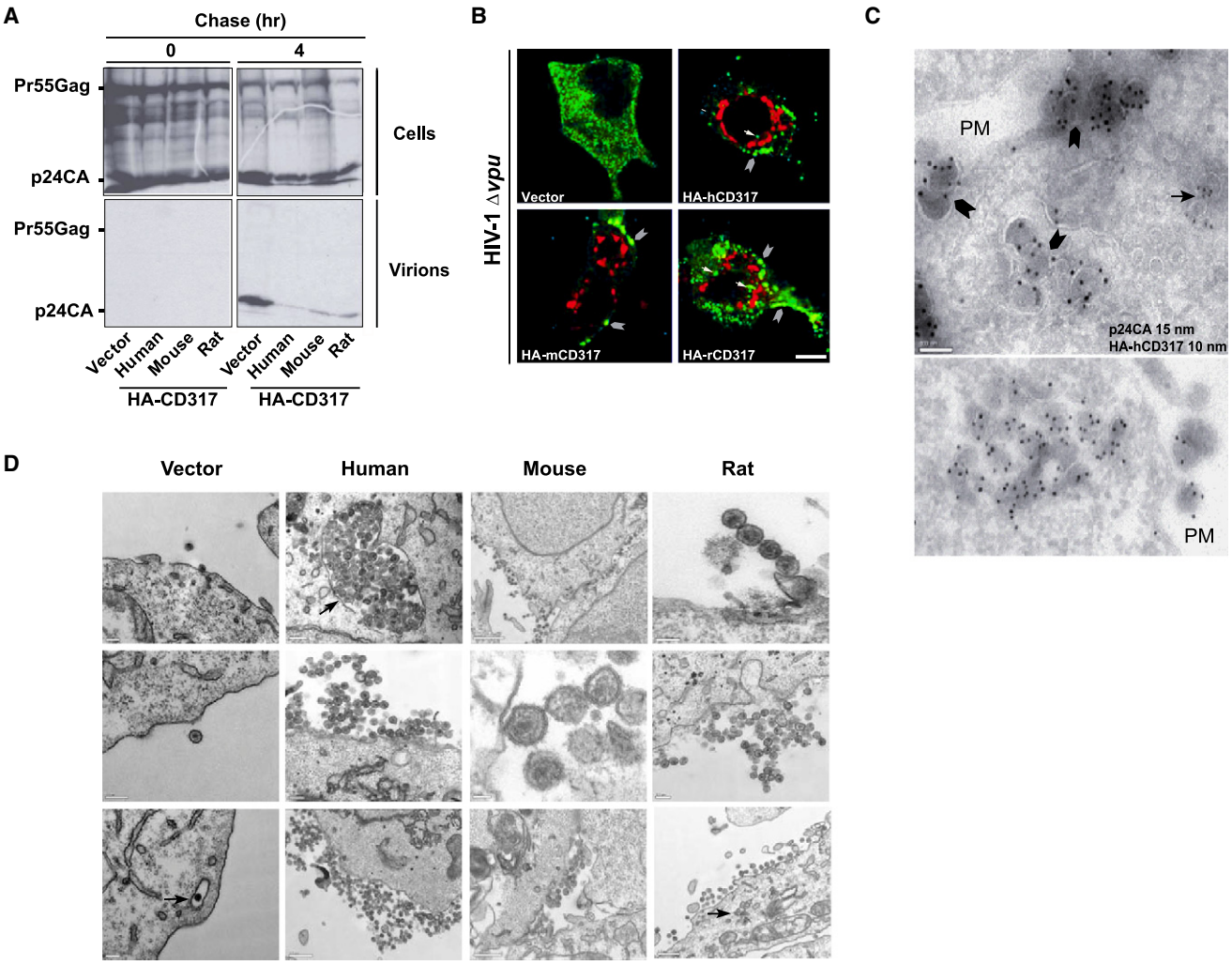

Figure 2. Impact of Human and Rodent CD317 on HIV-1 Gag by Pulse-Chase and Imaging Analyses

(A) Pulse-chase radiolabeling. 293T cells transiently transfected with psyn*gagpol* and expression plasmids for HA-CD317 or a vector control were pulse-labeled and then incubated with chase medium for 4 hr. After immunoprecipitation of cell lysates or supernatant with anti-p24CA antiserum, the proteins were separated by SDS-PAGE, and the gels were subjected to autoradiography. Shown are the results of one representative experiment out of three.

(B) Merged confocal micrographs for the localization of HIV-1 Gag (green, anti-p24CA) and HA-CD317 (red, anti-HA) in HIV-1Δ*vpu*-cotransfected 293T cells. Arrows indicates a typical intracellular (vesicular) Gag stain; arrow heads depict a typical plasma membrane Gag stain. Scale bar: 10 μm. Shown are the results of one representative experiment out of two.

(C) Electron microscopic localization of p24CA and HA-hCD317 in immunolabeled cryosections of 293T cells cotransfected with pHIV-1 WT (1.2 μg) and pcDNA3.1-HA-hCD317 (1.2 μg). Arrow heads indicate p24CA-labeled virions (15 nm gold); the arrow indicates vesicles labeled for HA-hCD317 (10 nm gold). PM: plasma membrane, scale bar: 200 nm.

(D) Electron microscopic images from epoxy resin-embedded sections of 293T cells cotransfected with pHIV-1Δ*vpu* (1.2 μg) and expression plasmids for the indicated HA-CD317 orthologs (1.2 μg) or a control vector. Arrows indicate intracellular accumulations of HIV-1 particles. Scale bars: 200 nm.

proviral DNA encoding HIV-1Δ*vpu* and constant, intermediate amounts of expression plasmids encoding HA-CD317. Under these experimental conditions, HA-hCD317 caused a pronounced restriction of HIV-1Δ*vpu* virion release. Importantly, the release of Vpu-defective HIV-1 could be rescued by *trans*-complemented Vpu in a concentration-dependent manner (Figure 3C). Notably, immunodetection of Vpu turned out to be rather nonlinear (Figure 3D), hampering quantitative assessment of the dose-effect relationship. Inhibition of virus release by CD317 from mouse and rat, however, could not be overcome by expression of Vpu (Figure 3C). This was true even at levels of Vpu expression that apparently exceeded those expressed by HIV-1 WT (Figure 3D).

The Sensitivity of CD317 to Vpu Antagonism Has a Complex Genetic Basis Involving All Three Major Structural Domains of the Restriction Factor

CD317 is an integral membrane protein with a highly unusual predicted topology: the amino-terminal cytoplasmic tail (CT) is followed by a single transmembrane domain (TM), an extracellular coiled-coil domain (ED), and a putative glycosyl phosphatidylinositol membrane anchor at the C terminus (Kupzig et al., 2003;

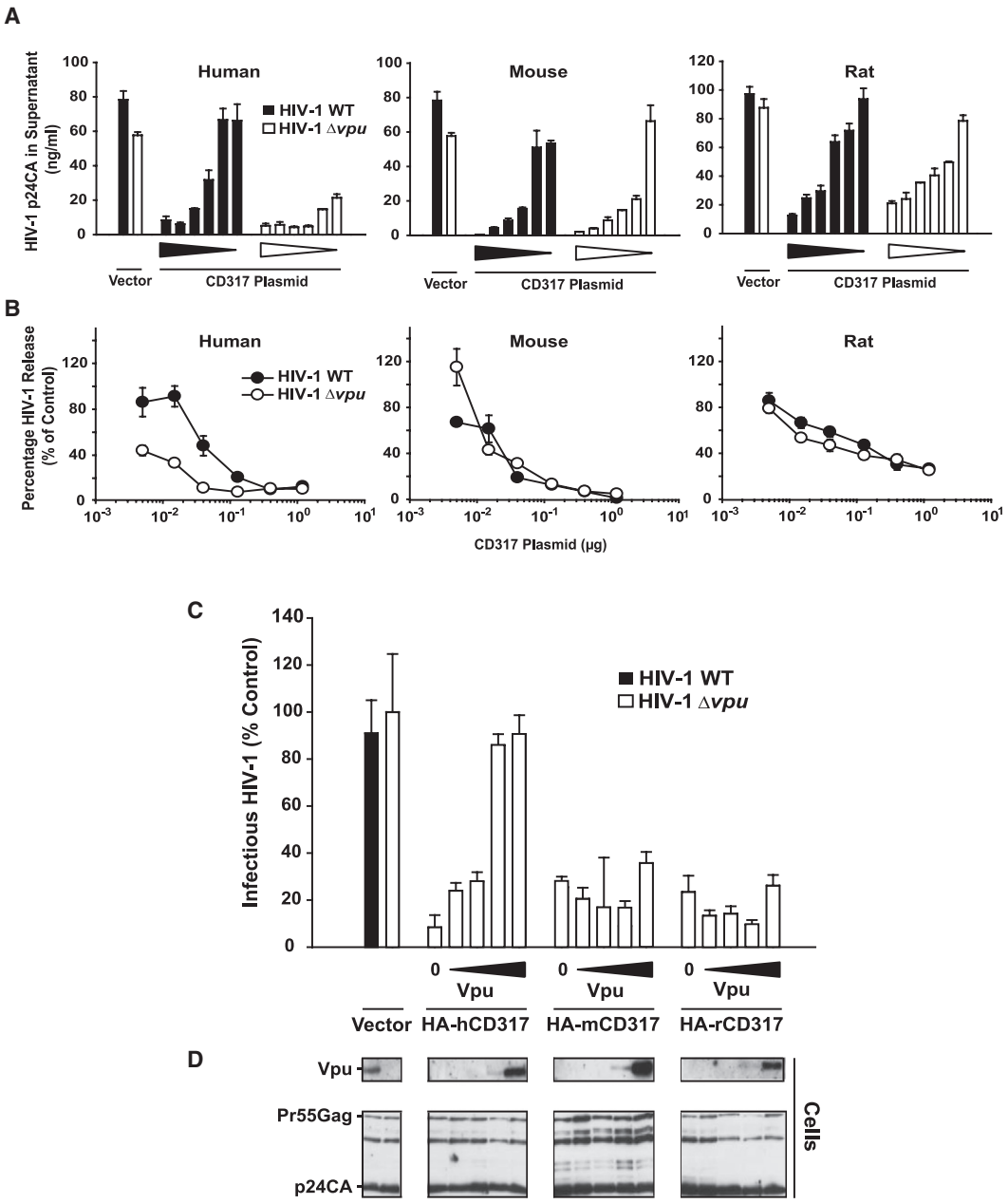

Figure 3. CD317 Inhibits HIV-1 Particle Release in a Concentration-Dependent Manner, and Vpu Counteracts the Activity of the Human Restriction Factor, but Not of the Rodent Orthologs

(A and B) Titration of plasmids encoding untagged human, mouse, or rat CD317 or no protein (vector) in 293T cells cotransfected with either pHIV-1 WT or pHIV-1Δ*vpu* and analyzed for the yield of virion-associated HIV-1 p24CA (A) and for effect on the percentage of HIV-1 release (B) as described in the legend to Figure 1. In (B), values obtained for vector-transfected controls were set to 100%. Values are arithmetic means ± SD from one representative experiment out of two.

(C and D) 293T cells were transfected with pHIV-1Δ*vpu* (1.2 μg, open bars), expression plasmids encoding HA-CD317 or HA alone (vector) (all 60 ng), and different concentrations of pcDNA-Vphu (0, 3, 13, 40, and 120 ng). The amount of proviral DNA as well as the total amount of DNA per transfection was kept constant. A transfection with pHIV-1 WT was included as reference (filled bar). The yield of infectious HIV-1 virions was quantified in a standardized TZM-bl reporter assay, and the vector-cotransfected HIV-1Δ*vpu* control was set to 100% (C). Histograms depict arithmetic means ± SD of triplicates. Western blot analyses of corresponding 293T cell lysates for Vpu or Gag proteins are shown (D). Values are arithmetic means ± SD from one representative experiment out of two.

Neil et al., 2008) (Figure 1A; insets in Figure 4). To map domains of the restriction factor that determine resistance or susceptibility to Vpu antagonism, we constructed a set of expression plasmids encoding HA-tagged CD317 chimeras with reciprocal exchanges of either the CT, TM, or ED between the human and rodent proteins (construct schematic in Figure S5).

All of these 12 chimeric CD317 proteins could be expressed in 293T cells, and the migration pattern of the chimeric proteins in

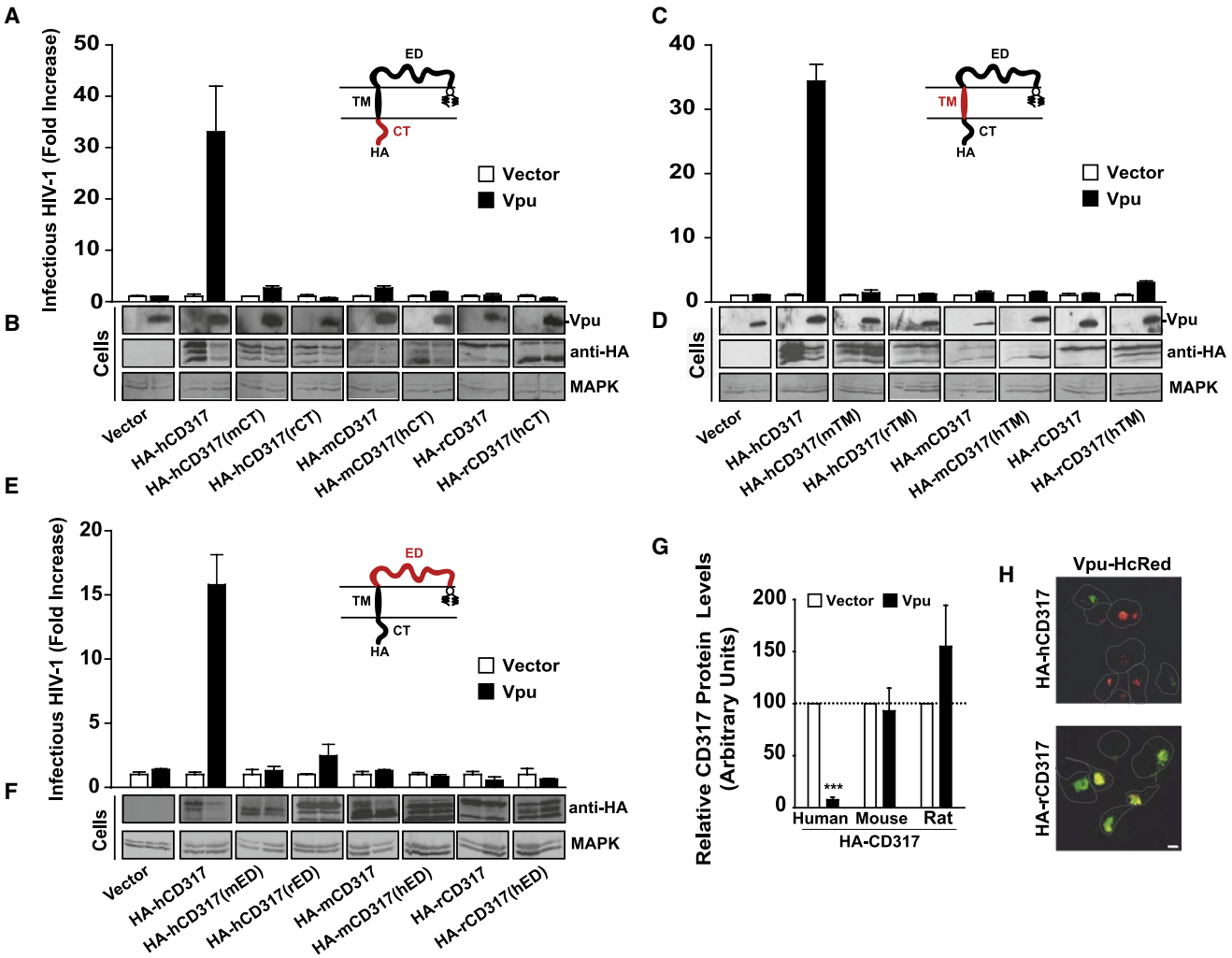

Figure 4. Critical Determinants for Vpu Counteraction Are Dispersed Across All Three Major Domains of CD317

(A–F) Effect of transient expression of wild-type, CT chimeric (A), TM chimeric (C), or ED chimeric (E) HA-CD317 proteins on infectious titers of Vpu-defective HIV-1 released by 293T cells cotransfected with pHIV-1Δ*vpu* (1.2 μg), expression plasmids encoding HA-CD317 or HA alone (vector), and either pcDNA-Vphu (120 ng) or an empty vector. The insets in (A), (C), and (E) depict the predicted topology of CD317 with the modified domain highlighted in red. Histogram bars give arithmetic means ± SD of triplicates. Also shown are western blot analyses for Vpu, HA-CD317, or MAPK in corresponding 293T cell lysates (B, D, and F).

(G) Protein levels of HA-CD317 proteins and MAPK were quantified by Odyssey Infrared Imaging System and Odyssey software. The ratio of values for HA-CD317 and MAPK for vector-cotransfected cells was set to 100%. Histogram bars depict the arithmetic means ± SEM of three independent experiments. ***$p < 0.001$ (Student's t test).

(H) Microscopic images of 293T cells cotransfected with expression plasmids for Vpu-HcRed and either human (top) or rat (bottom) HA-CD317 proteins. Cells were fixed and stained with anti-HA mAb HA.11, followed by Alexa 488-conjugated secondary antibodies (green). Yellow color in merged, low magnification images reflects cellular coexpression of Vpu-HcRed (red) and HA-CD317 (green). Scale bar: 10 μm.

SDS-PAGE analyses was complex (Figures 4B, 4D, 4F, and S6), which was primarily due to differential N-glycosylation (Figure S1). The subcellular distribution pattern of the human and rodent orthologs, as with many of the HA-CD317 chimeras, varied between numerous small vesicular structures and fewer larger accumulations (Figures S9 and S10). Upon coexpression with HIV-1Δ*vpu,* all CT, TM, and ED chimeras, as with the three wild-type HA-CD317 proteins, induced a redistribution of Gag into aggregates at the plasma membrane and/or intracellularly (Figure S10). Importantly, all 12 CT, TM, or ED chimeric HA-CD317 proteins potently inhibited the release of Vpu-defective HIV-1 at high expression levels of the restriction factor (Figure S6). The amount of plasmid required to achieve 50% inhibition of HIV-1 titers did not differ by more than 4-fold among the three wild-type CD317 proteins and most of the chimeric CD317 proteins, with the exception of four of these (Figure S7).

To determine susceptibility of the CD317 chimeras to Vpu antagonism, an experimental setup identical to that used to generate the data shown in Figures 3C and 3D was used. The amounts of cotransfected HA-CD317 plasmids were adjusted for each individual chimera to achieve a comparable degree of release restriction in the absence of Vpu. Remarkably, the inhibitory effect on release of HIV-1Δ*vpu* induced by all HA-CD317(CT) chimeras (Figures 4A and 4B), HA-CD317(TM)

chimeras (Figures 4C and 4D), and HA-CD317(ED) chimeras (Figures 4E and 4F) could not be counteracted by Vpu. This is consistent with the nearly superimposable titration curves for the restriction factors for wild-type and Vpu-defective HIV-1 (Figure S7). Specifically, transfer of the CT, TM, or ED from the rodent orthologs to HA-hCD317 resulted in resistance to the viral antagonist. Conversely, HA-rodent CD317 chimeras carrying the CT, TM, or ED from the human protein remained resistant to Vpu counteraction (Figures 4A, 4C, 4E, and S7). Collectively, this highlights a complex and apparently interdependent set of determinants located in the three domains of the human restriction factor that underlie its susceptibility to antagonism by the HIV-1 Vpu protein. Notably, of the 15 wild-type and human/rodent chimeric CD317 proteins analyzed, all displayed anti-HIV-1 activity, albeit with variable potency for some of the chimeras, but only the human protein could be antagonized by the action of Vpu. Thus, retroviral restriction appears to be a general and evolutionarily conserved feature of CD317, while antagonism by the Vpu protein of HIV-1 is specific for the human or possibly also other primate CD317 proteins.

Successful Antagonism by Vpu Correlates with the Depletion of Cellular Pools of HA-CD317

We noted that cellular levels of hCD317, but not of the rodent or chimeric CD317 proteins, were markedly reduced when Vpu was coexpressed (Figures 4B, 4D, and 4F). Quantitative western blot analyses with concurrent virological evaluation showed that the presence of Vpu resulted in a depletion of steady-state levels of HA-hCD317 by 93% relative to vector controls (Figure 4G). In contrast, expression of Vpu had no significant effect on mouse or rat CD317 protein levels (Figure 4G). This phenotype was corroborated by microscopic analysis of cells expressing Vpu-HcRed and HA-CD317. Expression of rodent CD317 protein (rat or mouse) was readily detectable in over 90% of Vpu-HcRed-positive 293T cells, whereas expression of hCD317 was detected in less than 20% of such cells (Figure 4H [low magnification image] and data not shown). Furthermore, a Vpu-dependent depletion of cellular pools of HA-CD317 was also seen in HA-hCD317-expressing 293T cells following infection with VSV-G pseudotyped HIV-1 (Figure S8). Therefore, the species-specific capacity of Vpu to functionally antagonize the CD317-mediated release restriction correlated with Vpu's ability to deplete cellular pools of the ectopically expressed restriction factor.

HIV-1 Depletes Endogenous hCD317 in Infected T Cells in a Vpu-Dependent Manner

Ectopic expression of HA-tagged CD317 in 293T cells may not faithfully recapitulate events occurring with the endogenous restriction factor. We therefore examined levels of native hCD317 in HIV-infected target cells, which constitutively express substantial levels of the intrinsic immunity factor, relative to the Vpu status and resulting virion release.

Productive infection was assessed by flow cytometry following intracellular p24CA staining. Infection of adherent TZM-bl cells (Figures 5A and 5B) and A3.01 T cells (Figures 5C and 5D) with HIV-1 WT induced a loss of hCD317 surface expression by 57% and 77%, respectively, relative to cells infected with HIV-1Δ*vpu*. This remarkable depletion of surface levels of CD317 by the WT virus coincided with a drastically enhanced release of infectious HIV-1 into the supernatant compared to the isogenic Vpu-defective counterpart, while the percentage of p24CA-positive cells in these cultures differed by only 1.5-fold (TZM-bl; Figure 5B) or 2-fold (A3.01; Figure 5D) at this early time point postinfection.

We further performed confocal immunofluoresence microscopy of the above HIV-1-infected A3.01 cultures to assess total cellular levels and distribution of hCD317 and Gag. Fifty-five percent of A3.01 T cells infected with HIV-1Δ*vpu* showed a strong accumulation of HIV-1 Gag (anti-p24CA staining) at the cell surface, consistent with virion tethering reported previously in this cell line (Klimkait et al., 1990) and, importantly, expressed CD317 at levels comparable to adjacent, uninfected cells (Figure 5F; 66% of Gag-positive cells coexpress CD317). Contrary to the results obtained with HA-hCD317 overexpressed in 293T cells, the endogenous protein localized, in addition to intracellular membranes, also to the plasma membrane and could frequently be detected at sites of p24CA accumulations. In contrast, HIV-1 WT-infected A3.01 T cells displayed a more dispersed cytoplasmic Gag distribution with little tethering (7% of all infected cells) and a significantly reduced frequency of CD317 expression (Figure 5E; 25% Gag-positive cells coexpress low levels of CD317). These results demonstrate that Vpu expressed in HIV-1_{NL4-3}-infected human T cells depletes the intrinsic immunity factor CD317 to overcome the naturally imposed cellular restriction to virion release.

Vpu Shortens the Half-Life of hCD317

To assess by which mechanism Vpu depletes cellular hCD317 species, [^{35}S]methionine pulse-chase radiolabeling studies were performed in 293T cells expressing HA-hCD317 together with Vpu or a control vector. In the absence of Vpu, the radiolabeled hCD317 protein showed a biphasic decay pattern: following a relatively quick disappearance of ~50% of the radiolabeled protein within 1 hr of chase, the remaining protein displayed a markedly higher stability (Figure 6A). In contrast, in the presence of Vpu, the radiolabeled hCD317 disappeared more rapidly, with a loss of ~85% within 1 hr and a complete disappearance by 3 hr of chase (Figure 6A). Notably, these experiments were conducted in the absence of other viral components, demonstrating that Vpu alone is sufficient to trigger the degradation of hCD317. Thus, Vpu achieves depletion of hCD317 by accelerating its degradation and hence shortening the half-life of the restriction factor.

Proteasome Inhibitors or the $Vpu_{S52AS56A}$ Mutant Block the Vpu-Mediated Depletion of hCD317, Resulting in a Restored Restriction of HIV-1 Release

We hypothesized that the proteasome might mediate the Vpu-induced degradation of hCD317. To test this, 293T cells were cotransfected with pHIV-1Δ*vpu* and pHA-hCD317 in the presence or absence of an expression plasmid encoding Vpu. Twenty-four hours after transfection, the medium was exchanged to remove virions produced up to this point and, subsequently, cells were treated for 18 hr with the proteasome inhibitors ALLN or *clasto*-lactacystin β-lactone or with DMSO alone. Notably, *clasto*-lactacystin β-lactone is a specific inhibitor of the 20S proteasome (Fenteany et al., 1995). At the end of the experiment, virions were purified through a sucrose cushion and quantified

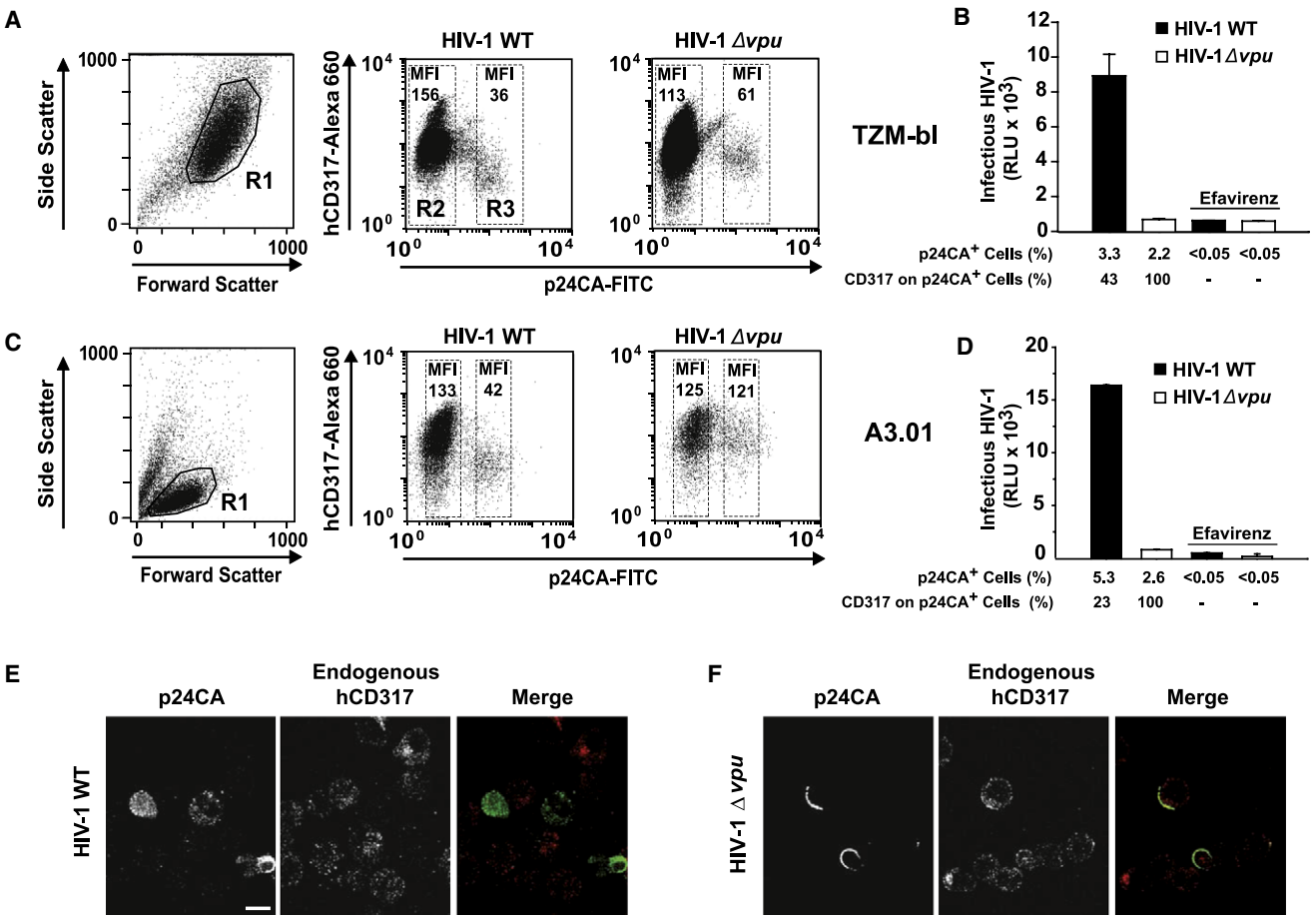

Figure 5. Expression of Endogenous hCD317 Is Lost from Cells Infected with HIV-1 WT, but Not from Cells Infected with HIV-1Δ*vpu*

(A–D) Human TZM-bl cells (A) or A3.01 T cells (T-lymphocytic cell line) (C) were infected with VSV-G-pseudotyped HIV-1 WT or HIV-1Δ*vpu* virus and 3–4 days postinfection analyzed by flow cytometry using side scattering of light (FSC/SSC) to identify live cells (gate R1), surface staining to quantify endogenous hCD317 expression levels, and concurrent intracellular p24CA staining to identify productively infected cells. At the time of cell harvest for flow cytometry, supernatant was removed to determine the yield of infectious HIV-1 (B and D). Efavirenz-treated cultures (1 μM) served as controls. Values represent arithmetic means ± SD of triplicates and originate from one of two similar experiments.

(E and F) Single channel and merged confocal micrographs for the localization of HIV-1 Gag (green, anti-p24CA) and endogenous hCD317 (red, anti-HM1.24/BST-2) in A3.01 T cells fixed in parallel to the analysis in (C). Scale bar: 10 μm.

by p24CA ELISA, and whole-cell lysates were subjected to western blot analysis. Treatment with both proteasome inhibitors completely abrogated the beneficial effect of Vpu on the release of HIV-1 particles (Figure 6B). Consistent with the idea that HIV-1 release inhibition is mediated by cellular levels of the restriction factor, HA-hCD317 steady-state levels were drastically elevated in cells treated with ALLN or *clasto*-lactacystin β-lactone when compared to DMSO-treated control cells, 9.3- and 9.2-fold, respectively (Figure 6C). Notably, in the absence of Vpu, both proteasome inhibitors reduced the production of HIV-1 ~2-fold (Figure 6B), consistent with a previous report (Schubert et al., 2000), and we observed a modest, 2-fold increase in HA-hCD317 levels (Figure 6C).

The Vpu2/6 mutant, which carries alanine substitutions of serine residues 52 and 56 within the cytoplasmic tail of the viral protein, has been reported to be impaired both in its ability to downregulate hCD317 from the cell surface (Van Damme et al., 2008) and to promote HIV-1 release in naturally restricted HeLa cells (Schubert et al., 1994; Van Damme et al., 2008). To directly probe the effect of this Vpu mutant on hCD317-mediated HIV-1 restriction and cell-associated hCD317 levels, wild-type Vpu or Vpu2/6 protein were coexpressed in 293T cells together with HIV-1Δ*vpu* and HA-hCD317. While Vpu expression readily overcame the release restriction and markedly depleted HA-hCD317 levels, the Vpu2/6 mutant failed to augment the release of Vpu-defective HIV-1 and also did not significantly impair HA-hCD317 expression (Figures 6D and 6E). Taken together, these results show that amino acid residues S52/S56 of Vpu and proteasome activity are critical for hCD317 antagonism by the viral protein, and that Vpu accelerates the physiological turnover of the intrinsic immunity factor by the proteasome.

The Ability of Vpu to Augment Virion Release and to Degrade hCD317 Strongly Correlate

Finally, we performed a Vpu dose-titration study to carefully assess the impact of the accessory protein's expression on

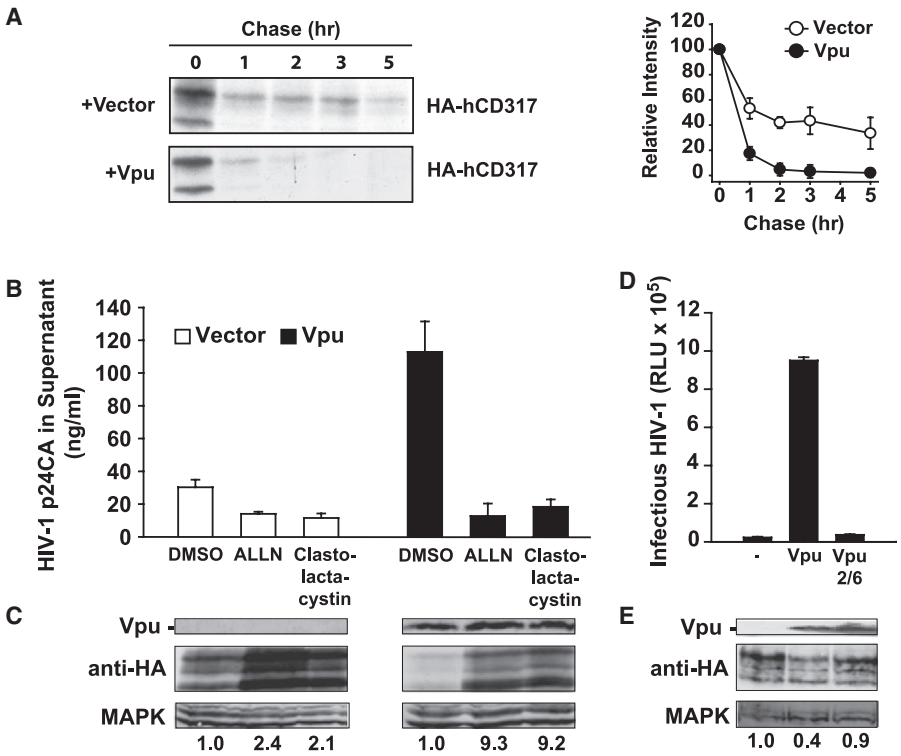

Figure 6. Vpu Depletes CD317 of Human Origin by Shortening Its Half-Life, Which Involves an Accelerated Degradation by the 20S Proteasome

(A) Pulse-chase radiolabeling. 293T cells transiently cotransfected with pHA-hCD317 together with an empty vector or pcDNA-Vphu were pulse-labeled and then incubated with chase media for the indicated time points. After immunoprecipitation with anti-HA antibody, proteins were separated by SDS-PAGE, and the gels were subjected to autoradiography (left panel). The relative intensity of the HA-hCD317 bands was quantified, and values at 0 hr were set to 100 and graphed. Given are the arithmetic means ± SD of three independent experiments (right panel).

(B and C) Proteasome inhibitor study. 293T cells were cotransfected with pHIV-1Δ*vpu* together with pcDNA-Vphu or an empty control vector. Twenty-four hours after transfection, the medium was exchanged to remove virions produced up to this point. Cells were then cultivated for 2 hr in the presence of the proteasome inhibitors ALLN (25 μM) or *clasto*-lactacystin β-lactone (20 μM) or with DMSO alone (0.25%), washed again, and then treated for an additional 16 hr in the presence of drugs until harvest. Shown are virion-associated p24CA levels in supernatants (B) and western blot analyses of corresponding 293T cell lysates for Vpu, HA-hCD317, and MAPK with quantified ratios for HA-hCD317/MAPK signals given below (DMSO-treated vector controls set to 1.0) (C). Values represent arithmetic means ± SD of triplicates and originate from one representative experiment out of two.

(D and E) Vpu mutant study. 293T cells were transfected to express HIV-1Δ*vpu* and HA-hCD317 together with either wild-type Vpu ("Vpu") or a mutant, in which S52 and S56 are replaced by alanine ("Vpu2/6"). Two days posttransfection, the yield of infectious HIV-1 (D) and levels of Vpu, HA-hCD317, and MAPK (E) were analyzed. The percentage of HA-hCD317/MAPK expression relative to Vpu-negative control cells, set to 1.0, is given. Values represent arithmetic means ± SD of triplicates and originate from one of two similar experiments.

HIV-1 release in relation to its ability to deplete levels of hCD317 in virus-producing cells. To this end, 293T cells were cotransfected with a constant, intermediate amount of pHA-hCD317 and pHIV-1Δ*vpu* together with decreasing amounts of the expression plasmid encoding Vpu. In addition, a cotransfection with pHIV-1 WT was included. Culture supernatants and whole-cell lysates were analyzed 48 hr later.

Coexpression of HA-hCD317 with HIV-1 WT (but not with Vpu-defective HIV-1) allowed, as expected, high-level release of infectious virions (Figure 7A). This was accompanied by a marked depletion of HA-hCD317 in 293T producer cells (Figure 7B). For Vpu-defective HIV-1, virion release was rescued by coexpression of Vpu in a concentration-dependent manner, and also a concentration-dependent depletion of cellular HA-hCD317 was observed (Figures 7A and 7B). Importantly, a more refined correlative and quantitative analysis of these two Vpu-modulated parameters revealed that depletion of the restriction factor (Figure 7C, filled circles) and values for release of infectious HIV-1 (Figure 7C, open triangles) closely matched across the entire range of coexpressed Vpu concentrations. Moreover, replotting of values for these two parameters, i.e., infectious HIV-1 release and HA-hCD317 depletion, from Figure 7C demonstrated a strong positive correlation (Figure 7D, open squares) (Pearson's correlation coefficient: r = 0.94; p = 0.0005). Of note, plotting of values for these two parameters obtained for HIV-1 WT (Figures 7A and 7B) fit well into this quantitative relationship established for Vpu-defective HIV-1 in the context of *trans*-complemented Vpu (Figure 7D, filled square). Collectively, these findings provide strong evidence for an important mechanistic role of CD317 degradation by Vpu from HIV-1_{NL4-3} for the accessory protein's ability to overcome the restriction of the intrinsic immune factor in human cells.

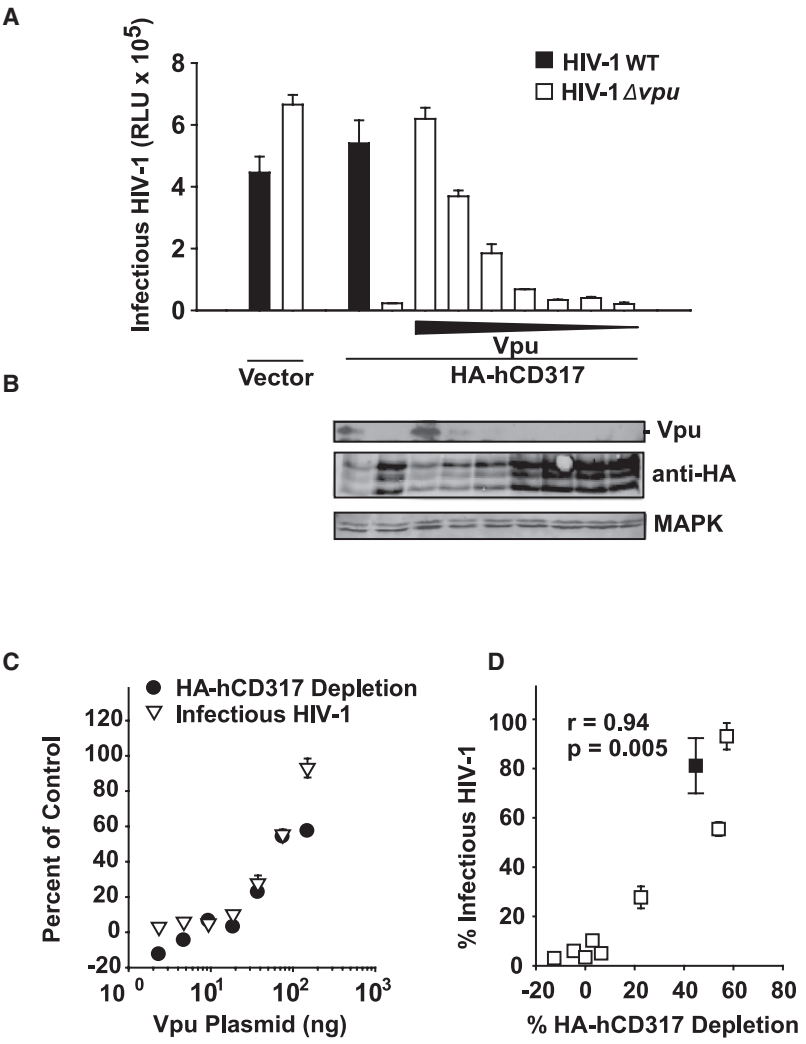

Figure 7. The Ability of Vpu to Deplete CD317 Closely Correlates with its Capacity to Enhance HIV-1 Release

(A and B) 293T cells were transfected by calcium phosphate precipitation with three different plasmids: (1) pHA-hCD317 (100 ng), (2) either pHIV-1 WT or pHIV-1Δ*vpu* (1.2 μg), (3) different amounts of pcDNA-Vphu (0, 150, 75, 37.5, 18.8, 9.4, 4.7, or 2.3 ng). As a reference, pHIV-1Δ*vpu* or pHIV-1 WT were also expressed in cells in the absence of *trans*-complemented Vpu and HA-hCD317. Forty-eight hours later, supernatants and whole-cell lysates were analyzed for HIV-1 infectivity (A) and relative levels of HA-hCD317 and Vpu, respectively (B), as detailed above. Values represent arithmetic means ± SD of triplicates.

(C) Quantification and correlative analysis of infectious HIV-1 release (A) and HA-hCD317 depletion (B) upon Vpu titration. Open triangles: the percentage of HIV-1Δ*vpu* virions secreted in the presence of HA-hCD317 and different amounts of *trans*-complemented Vpu are given relative to HIV-1Δ*vpu* levels secreted in the absence of Vpu and HA-hCD317, which was set to 100% (data taken from [A]). Filled circles: the percentage of HA-hCD317 depletion is given with relative levels for lysates of HIV-1Δ*vpu*- and HA-hCD317-transfected cells in the absence of Vpu set to 0% depletion (data derived from [B]).

(D) Relative levels of HA-hCD317 depletion and released HIV-1 for different Vpu concentrations from (C) plotted against each other (open squares). For these values, the Pearson's correlation coefficient r and the corresponding p value were calculated using GraphPad Software (indicated in [D]). The filled square depicts the value obtained for HIV-1 WT in (A) and (B).

DISCUSSION

Retroviruses display species-specific patterns of infection and pathogenesis. The impairment of retroviral replication in foreign and sometimes even in cognate hosts stems, in part, from the action of host-encoded restriction factors. These cellular factors can interfere with distinct steps of the replication cycle of diverse viral pathogens and are thus an important arm of the innate defense system. Many retroviruses have, in turn, evolved gene products that can inactivate or overcome these intrinsic antiviral barriers by diverse strategies that at present are frequently still poorly understood. Here, we report that HIV-1 has evolved its Vpu gene product to antagonize the intrinsic immunity factor CD317 in human but not in rodent cells. This species-specific antagonism involves the Vpu-mediated depletion of cellular pools of hCD317 by targeting the restriction factor for degradation by the 20S proteasome.

For Vpu-defective HIV-1, expression of CD317 from human, mouse, or rat induced a virion release block that was characterized by an accumulation of mature particles tethered to the cell surface or to each other, as well as by virion clusters in seemingly intracellular compartments. Despite their profound sequence heterogeneity, human and rodent CD317 orthologs therefore exert comparable anti-HIV-1 activities by targeting the virion release step, presumably by a common mechanism. The antiretroviral activity of CD317 thus appears to be evolutionarily conserved, regardless of whether the viral pathogen naturally infects the species.

The molecular mechanism by which CD317 inhibits release of retroviral particles from productively infected cells is still unclear. Based on its highly unusual predicted topology, ability to form dimers via disulfide bonds, and colocalization of HA-hCD317 and Gag.GFP in 293T cells, CD317 has been suggested to bridge cell-virion or virion-virion interactions by placing one of its two lipid-anchoring domains in each membrane or by dimerization of two juxtaposed CD317 molecules (Neil et al., 2008).

Our analyses of 293T cells presented herein, however, did not provide evidence for a physical involvement of CD317. Confocal and immunoelectron microscopy failed to identify substantial colocalization between HIV-1 Gag and ectopically expressed HA-CD317, while antiviral activity of the restriction factor was readily exerted. CD317, detected through an intracytoplasmic N-terminal HA-epitope tag, predominantly localized to intracellular membranes and not to the cell surface, from which virions bud, and no enriched labeling was detected on virions and budding sites. On the contrary, endogenous hCD317 in human A30.1 T cells, detected through an epitope in the ED (Ishikawa et al., 1995), was found more prominently at the plasma

membrane and in Gag-positive "virion tethers" in HIV-1Δ*vpu*-infected cells. These puzzling results warrant future studies into the mechanism by which CD317 affects HIV-1 particle release in different cell types.

Independent of the subcellular localization of CD317 action, it seems counterintuitive that the apparent virion release defect with accumulations of tethered virions at the plasma membrane does not lead to increased levels of cell-associated p24CA, as also noted in two recent studies (Neil et al., 2008; Van Damme et al., 2008). However, it is unclear what fraction of the total amount of cell-associated p24CA is truly reflected by these morphologically striking virion aggregates and, in addition, Bieniasz and colleagues have reported an enhanced endocytosis rate of tethered virions and accumulation in endosomes prior to targeting for degradation (Neil et al., 2006). Together, this may contribute to a balance between the enhancement (tethering) and reduction (degradation) of cell-associated p24CA levels in CD317-expressing cells, and future mechanistic studies should address this in more detail.

In contrast to the conserved anti-HIV-1 activities of human and rodent CD317 proteins, viral antagonism of these orthologs was species-specific. A comprehensive set of interspecies CD317 chimeras revealed that the rodent-specific resistance as well as the human-specific susceptibility to Vpu antagonism have a complex genetic basis and are governed by determinants in the CT, TM, and ED of the intrinsic immunity factor. Furthermore, the species-specific functional antagonism of Vpu correlated with the ability of Vpu to modulate cellular pools of the respective restriction factor. This is in contrast to an experiment presented by Neil and colleagues (Neil et al., 2008), which did not show altered HA-hCD317 levels upon Vpu coexpression. In our study, the reduction of steady-state levels of hCD317 by Vpu was demonstrated by quantitative western blot analyses, in line with initial observations by Bartee and colleagues (Bartee et al., 2006). In addition, confocal microscopy revealed a profound reduction in the number of cells expressing HA-hCD317 upon coexpression of Vpu-HcRed or upon infection with HIV-1 WT, but not HIV-1Δ*vpu*. Furthermore, productive HIV-1_{NL4-3} infection of human A3.01 T cells led to marked reduction of cell surface levels and intracellular pools of endogenous CD317. In contrast, levels of rodent CD317 proteins were unaltered by coexpression of Vpu. Mouse and rat CD317 are thus resistant to Vpu counteraction and act as potent inhibitors of wild-type HIV-1 infection.

We propose that proteasomal degradation of CD317 lies at the heart of the action of Vpu from HIV-1_{NL4-3} and is the key mechanism by which the wild-type virus overcomes the restriction of virion release in infected human T cells. In support of this notion, this manuscript presents the following findings: first, Vpu heavily impairs the intracellular stability of hCD317. Second, efficacies of HIV-1 release rescue and depletion of hCD317 in Vpu titration studies were strictly correlated. Also of interest in this context, overexpression of hCD317 potently inhibited the release of Vpu-expressing HIV-1 WT, demonstrating that the viral antagonism of the "Vpu-sensitive" human restriction factor is saturable. Third, the Vpu-induced degradation of hCD317 involves the proteasome, since addition of proteasomal inhibitors completely blocked the ability of Vpu to deplete CD317 and effectively eliminated the Vpu-induced rescue of virion release. Fourth, the Vpu2/6 mutant, which fails to promote HIV-1 release in restricted HeLa cells (Schubert et al., 1994; Van Damme et al., 2008), neither depleted hCD317 nor overcame the virion release restriction. This mapping provides an initial insight into possible similarities between the pathways by which Vpu targets the viral entry receptor CD4 and the innate immunity factor CD317 for proteasomal degradation, i.e., via pathways involving β-TrCP and E3 ubiquitin ligases (Malim and Emerman, 2008). Fifth, Vpu expressed in HIV-1_{NL4-3}-infected human T cells depleted hCD317 and overcame the naturally imposed cellular restriction to virion release. Finally, the rodent orthologs were resistant to both Vpu-mediated degradation and release rescue. Collectively, these findings provide strong evidence for the mechanistic importance of Vpu-mediated CD317 degradation for the accessory protein's ability to overcome the restriction imposed by the intrinsic immunity factor. At this point, additional, degradation-independent effects of Vpu on virion release in the context of CD317 expression, as well as Vpu allele-specific effects, cannot be excluded.

Adding another level of complexity to the restriction of retroviral release by the intrinsic immune system, human calcium-modulating cyclophilin ligand (CAML) was recently identified by Varthakavi et al. as a second Vpu-sensitive host restriction factor that inhibits the release of HIV from human cells (Varthakavi et al., 2008). Thus, humans express at least two structurally unrelated cellular proteins that either coordinate or directly mediate restriction at the final step in the retroviral replication cycle. The combined action of hCD317 and hCAML may conceivably account for the long-known dominant cellular restriction phenotype to HIV release.

Species-specific cellular factors like CD317 impose barriers to retroviral infection. The fact that Vpu from HIV-1 can successfully interfere with the intrinsic antiviral action of CD317 in humans, but not in rodents, hints at the evolutionary importance of this ability. This species-specific activity may not only oppose zoonotic transmission—reflected, in part, also by difficulties in establishing high-viremic rodent models of HIV disease—but provides an important resource for understanding fundamental principles of virus-host interaction. Insight from the current study into HIV-1 Vpu and previous work on HIV-1 Vif, which also depletes intracellular pools of its cellular counterplayer APOBEC3G in a species-specific fashion (Browne and Littman, 2008; Goila-Gaur and Strebel, 2008; Mariani et al., 2003; Stopak et al., 2003), highlight that the struggle over expression levels of intrinsic immunity factors is a general theme of virus-host coevolution. The antiviral potency combined with the Vpu resistance of rodent CD317 identified herein will guide in vivo strategies for the advancement of transgenic small animal models of HIV infection (Keppler et al., 2002). Furthermore, the Vpu-CD317/CAML axis in the context of species-specific interaction patterns forms a potential target for the development of novel anti-HIV drugs.

EXPERIMENTAL PROCEDURES

Plasmids

pCMV-SPORT6-hBST2/hCD317 and pCMV-Sport6-mBST2/mCD317 were from Open Biosystems (Huntsville, AL). pCMV-SPORT6-rCD317 was generated by subcloning of rBST2 cDNA (Kupzig et al., 2003) into pCMV.SPORT6 with EcoRI. pcDNA3.1/*neo*-based expression vectors for CD317 from all three species containing amino-terminal HA-epitope tags were generated by PCR. Human-rodent HA-CD317 CT, TM, or ED domain chimeras were designed

with Clone Manager based on topological predictions (Kupzig et al., 2003) (see also Figures 1A, 4A, 4C, 4E, S6, and S7), and corresponding oligonucleotide fragments were synthesized by Geneart. Fragments containing rodent CT, TM, or ED domains in the context of hCD317 were subcloned into pcDNA3.1-HA-hCD317 with KpnI and BbvCI. Fragments containing the CT, TM, or ED domain of human origin in the context of pcDNA3.1-HA-mCD317 were subcloned with KpnI and BsmBI. The subcloning of the CT, TM, or ED domains from humans in the context of pcDNA3.1-HA-rCD317 was carried out with KpnI and PpuMI or SgrAI and PpuMI, respectively. The subviral plasmid psyn*gagpol*, encoding codon-optimized *gag* and *pol* from HIV-1_{NL4-3}, was from Ralf Wagner (University of Regensburg, Germany). The plasmid pcDNA-Vphu, expressing a codon-optimized, Rev-independent HIV-1_{NL4-3} Vpu protein (Nguyen et al., 2004), was from Klaus Strebel (National Institutes of Health; Bethesda, MD), and pVpu-HcRed encoding this codon-optimized Vpu fused to HcRed was from Paul Spearman (Emory University School of Medicine; Atlanta) (Varthakavi et al., 2006). The Vpu S52AS56A mutant ("Vpu 2/6") was generated by site-directed mutagenesis from pcDNA-Vphu.

Viruses

Proviral plasmids pHIV-1_{NL4-3} WT (BH10 Env) and pHIV-$1_{NL4-3}\Delta$*vpu* (BH10 Env) (Pfeiffer et al., 2006) were from Valerie Bosch (Deutsches Krebsforschungszentrum; Heidelberg, Germany). Supernatants from provirus-transfected cells were harvested on day 2 posttransfection, and virions pelleted through a 20% sucrose cushion by ultracentrifugation (44,000 *g*, 4°C, 60 min) and resuspended in PBS (infectivity analysis) or 1% Triton X-100/PBS (immunoblotting or p24CA ELISA). Pellets of transfected cells were washed in PBS and lysed in SDS-lysis buffer for immunoblot or 1% Triton X-100/PBS for p24CA ELISA. The virion- and cell-associated amount of HIV-1 p24CA antigen was determined by an antigen enzyme-linked immunosorbent assay (p24CA ELISA) (Keppler et al., 2005). Of note, this ELISA is at least 1000-fold less sensitive for the detection of Pr55Gag compared to p24CA (H.-G.K., unpublished data).

Cells and Transfections

All cell lines were obtained from the American Type Culture Collection and cultivated under standard condition in Dulbecco's modified Eagle's medium or RPMI1640 supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin, and 1% L-glutamine (all from Invitrogen; Carlsbad, CA). 293T cells were transfected by calcium phosphate DNA precipitation or FuGENE 6 (Roche Diagnostics; Risch, Switzerland).

HIV-1 Infectivity Assay

The infectivity of HIV-1 was determined 48 hr after infection in a standardized 96-well titration assay on TZM-bl cells by luminometric analysis of firefly luciferase activity (Geuenich et al., 2008).

Confocal Immunofluorescence Microscopy

Transfected cells growing on coverslips were fixed with 4% paraformaldehyde and permeabilized for 2 min with 0.1% Triton X-100 in PBS. Cells were blocked for 1 hr with 1% bovine serum albumin in PBS and stained for p24CA and HA using appropriate primary and secondary antibodies as described (Fackler et al., 2006; Hannemann et al., 2008). Coverslips were mounted in mounting medium (Dianova; Hamburg, Germany) and analyzed with a Zeiss LSM 510 confocal microscope with a 100× PLAN-APO objective lens. Images were recorded with the Zeiss proprietary software LSM 5 and processed with Adobe Photoshop 6.0. Gag localization was classified for individual cells in principle as reported (Neil et al., 2008).

Proteasome Inhibitors

ALLN and *clasto*-lactacystin β-lactone were purchased from Calbiochem (San Diego, CA) and used as detailed in the legend to Figure 6.

Immunoblotting

Cells were lysed in either buffer A (50 mM HEPES, 135 mM NaCl, 10% glycerol, 1% Triton X-100, 1 mM EDTA) or protease inhibitor cocktail (Sigma; St. Louis), pH 7.2, for 1 hr at 4°C. Lysates were collected after centrifugation at 13,200× *g* for 20 min at 4°C and analyzed for protein concentration using the BCA protein assay (Pierce; Rockford, IL). Alternatively, cell pellets were directly resuspended in SDS-lysis buffer. Proteins were run on a 10% or 12.5% SDS-PAGE and transferred onto nitrocellulose. Blocked membranes were probed with the following primary antisera/antibodies: rabbit polyclonal serum anti-HIV-1 p24CA, anti-Vpu (from Eric Cohen, Institut de recherches cliniques de Montréal), anti-MAPK (Santa Cruz Biotechnology; Santa Cruz, CA), as well as mouse anti-HA mAb HA.11 (Covance; Princeton, NJ). Secondary antibodies were conjugated to either horseradish peroxidase for ECL-based detection or Alexa 700/800 fluorescent dyes for detection by Odyssey Infrared Imaging System (LI-COR Biosciences; Lincoln, NE) and quantification by Odyssey software (version 2.1).

Supplemental Data report details on electron microscopy, HIV-1 Gag and hCD317 pulse-chase analysis, flow cytometric analysis of infected cells, and immunoprecipitation and deglycosylation of HA-CD317 chimeras.

SUPPLEMENTAL DATA

Supplemental Data include Supplemental Experimental Procedures, Supplemental References, and ten figures and can be found online at http://www.cell.com/cellhostandmicrobe/supplemental/S1931-3128(09)00062-6.

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REFERENCES

Bartee, E., McCormack, A., and Früh, K. (2006). Quantitative membrane proteomics reveals new cellular targets of viral immune modulators. PLoS Pathog. *2*, e107.

Bieniasz, P.D. (2004). Intrinsic immunity: a front-line defense against viral attack. Nat. Immunol. *5*, 1109–1115.

Blasius, A.L., Giurisato, E., Cella, M., Schreiber, R.D., Shaw, A.S., and Colonna, M. (2006). Bone marrow stromal cell antigen 2 is a specific marker of type I IFN-producing cells in the naive mouse, but a promiscuous cell surface antigen following IFN stimulation. J. Immunol. *177*, 3260–3265.

Bour, S., Schubert, U., Peden, K., and Strebel, K. (1996). The envelope glycoprotein of human immunodeficiency virus type 2 enhances viral particle release: a Vpu-like factor? J. Virol. *70*, 820–829.

Bour, S.P., Aberham, C., Perrin, C., and Strebel, K. (1999). Lack of effect of cytoplasmic tail truncations on human immunodeficiency virus type 2 ROD env particle release activity. J. Virol. *73*, 778–782.

Browne, E.P., and Littman, D.R. (2008). Species-specific restriction of apobec3-mediated hypermutation. J. Virol. *82*, 1305–1313.

Chiu, Y.L., and Greene, W.C. (2008). The APOBEC3 cytidine deaminases: an innate defensive network opposing exogenous retroviruses and endogenous retroelements. Annu. Rev. Immunol. *26*, 317–353.

Fackler, O.T., Moris, A., Tibroni, N., Giese, S.I., Glass, B., Schwartz, O., and Kräusslich, H.G. (2006). Functional characterization of HIV-1 Nef mutants in the context of viral infection. Virology *351*, 322–339.

Fenteany, G., Standaert, R.F., Lane, W.S., Choi, S., Corey, E.J., and Schreiber, S.L. (1995). Inhibition of proteasome activities and subunit-specific amino-terminal threonine modification by lactacystin. Science *268*, 726–731.

Geuenich, S., Goffinet, C., Venzke, S., Nolkemper, S., Baumann, I., Plinkert, P., Reichling, J., and Keppler, O.T. (2008). Aqueous extracts from peppermint,

sage and lemon balm leaves display potent anti-HIV-1 activity by increasing the virion density. Retrovirology *5*, 27.

Goff, S.P. (2004). Retrovirus restriction factors. Mol. Cell *16*, 849–859.

Goila-Gaur, R., and Strebel, K. (2008). HIV-1 Vif, APOBEC, and intrinsic immunity. Retrovirology *5*, 51.

Goto, T., Kennel, S.J., Abe, M., Takishita, M., Kosaka, M., Solomon, A., and Saito, S. (1994). A novel membrane antigen selectively expressed on terminally differentiated human B cells. Blood *84*, 1922–1930.

Göttlinger, H.G., Dorfman, T., Cohen, E.A., and Haseltine, W.A. (1993). Vpu protein of human immunodeficiency virus type 1 enhances the release of capsids produced by gag gene constructs of widely divergent retroviruses. Proc. Natl. Acad. Sci. USA *90*, 7381–7385.

Hannemann, S., Madrid, R., Stastna, J., Kitzing, T., Gasteier, J., Schönichen, A., Bouchet, J., Jimenez, A., Geyer, M., Grosse, R., et al. (2008). The Diaphanous-related Formin FHOD1 associates with ROCK1 and promotes Src-dependent plasma membrane blebbing. J. Biol. Chem. *283*, 27891–27903.

Iida, S., Fukumori, T., Oshima, Y., Akari, H., Koyama, A.H., and Adachi, A. (1999). Compatibility of Vpu-like activity in the four groups of primate immunodeficiency viruses. Virus Genes *18*, 183–187.

Ishikawa, J., Kaisho, T., Tomizawa, H., Lee, B.O., Kobune, Y., Inazawa, J., Oritani, K., Itoh, M., Ochi, T., Ishihara, K., et al. (1995). Molecular cloning and chromosomal mapping of a bone marrow stromal cell surface gene, BST2, that may be involved in pre-B-cell growth. Genomics *26*, 527–534.

Keppler, O.T., Welte, F.J., Ngo, T.A., Chin, P.S., Patton, K.S., Tsou, C.L., Abbey, N.W., Sharkey, M.E., Grant, R.M., You, Y., et al. (2002). Progress toward a human CD4/CCR5 transgenic rat model for de novo infection by human immunodeficiency virus type 1. J. Exp. Med. *195*, 719–736.

Keppler, O.T., Allespach, I., Schüller, L., Fenard, D., Greene, W.C., and Fackler, O.T. (2005). Rodent cells support key functions of the human immunodeficiency virus type 1 pathogenicity factor Nef. J. Virol. *79*, 1655–1665.

Klimkait, T., Strebel, K., Hoggan, M.D., Martin, M.A., and Orenstein, J.M. (1990). The human immunodeficiency virus type 1-specific protein Vpu is required for efficient virus maturation and release. J. Virol. *64*, 621–629.

Kupzig, S., Korolchuk, V., Rollason, R., Sugden, A., Wilde, A., and Banting, G. (2003). Bst-2/HM1.24 is a raft-associated apical membrane protein with an unusual topology. Traffic *4*, 694–709.

Malim, M.H., and Emerman, M. (2008). HIV-1 accessory proteins—ensuring viral survival in a hostile environment. Cell Host Microbe *3*, 388–398.

Mariani, R., Chen, D., Schröfelbauer, B., Navarro, F., Konig, R., Bollman, B., Munk, C., Nymark-McMahon, H., and Landau, N.R. (2003). Species-specific exclusion of APOBEC3G from HIV-1 virions by Vif. Cell *114*, 21–31.

Neil, S.J., Eastman, S.W., Jouvenet, N., and Bieniasz, P.D. (2006). HIV-1 Vpu promotes release and prevents endocytosis of nascent retrovirus particles from the plasma membrane. PLoS Pathog. *2*, e39.

Neil, S.J., Sandrin, V., Sundquist, W.I., and Bieniasz, P.D. (2007). An interferon-alpha-induced tethering mechanism inhibits HIV-1 and Ebola virus particle release but is counteracted by the HIV-1 Vpu protein. Cell Host Microbe *2*, 193–203.

Neil, S.J., Zang, T., and Bieniasz, P.D. (2008). Tetherin inhibits retrovirus release and is antagonized by HIV-1 Vpu. Nature *451*, 425–430.

Nguyen, K.L., Llano, M., Akari, H., Miyagi, E., Poeschla, E.M., Strebel, K., and Bour, S. (2004). Codon optimization of the HIV-1 vpu and vif genes stabilizes their mRNA and allows for highly efficient Rev-independent expression. Virology *319*, 163–175.

Noble, B., Abada, P., Nunez-Iglesias, J., and Cannon, P.M. (2006). Recruitment of the adaptor protein 2 complex by the human immunodeficiency virus type 2 envelope protein is necessary for high levels of virus release. J. Virol. *80*, 2924–2932.

Pfeiffer, T., Pisch, T., Devitt, G., Holtkotte, D., and Bosch, V. (2006). Effects of signal peptide exchange on HIV-1 glycoprotein expression and viral infectivity in mammalian cells. FEBS Lett. *580*, 3775–3778.

Ritter, G.D., Jr., Yamshchikov, G., Cohen, S.J., and Mulligan, M.J. (1996). Human immunodeficiency virus type 2 glycoprotein enhancement of particle budding: role of the cytoplasmic domain. J. Virol. *70*, 2669–2673.

Sakuma, R., Noser, J.A., Ohmine, S., and Ikeda, Y. (2007). Rhesus monkey TRIM5alpha restricts HIV-1 production through rapid degradation of viral Gag polyproteins. Nat. Med. *13*, 631–635.

Schubert, U., Henklein, P., Boldyreff, B., Wingender, E., Strebel, K., and Porstmann, T. (1994). The human immunodeficiency virus type 1 encoded Vpu protein is phosphorylated by casein kinase-2 (CK-2) at positions Ser52 and Ser56 within a predicted alpha-helix-turn-alpha-helix-motif. J. Mol. Biol. *236*, 16–25.

Schubert, U., Clouse, K.A., and Strebel, K. (1995). Augmentation of virus secretion by the human immunodeficiency virus type 1 Vpu protein is cell type independent and occurs in cultured human primary macrophages and lymphocytes. J. Virol. *69*, 7699–7711.

Schubert, U., Bour, S., Willey, R.L., and Strebel, K. (1999). Regulation of virus release by the macrophage-tropic human immunodeficiency virus type 1 AD8 isolate is redundant and can be controlled by either Vpu or Env. J. Virol. *73*, 887–896.

Schubert, U., Ott, D.E., Chertova, E.N., Welker, R., Tessmer, U., Princiotta, M.F., Bennink, J.R., Kräusslich, H.G., and Yewdell, J.W. (2000). Proteasome inhibition interferes with gag polyprotein processing, release, and maturation of HIV-1 and HIV-2. Proc. Natl. Acad. Sci. USA *97*, 13057–13062.

Sebastian, S., and Luban, J. (2007). The Retroviral Restriction Factor TRIM5alpha. Curr. Infect. Dis. Rep. *9*, 167–173.

Simon, J.H., Gaddis, N.C., Fouchier, R.A., and Malim, M.H. (1998). Evidence for a newly discovered cellular anti-HIV-1 phenotype. Nat. Med. *4*, 1397–1400.

Stopak, K., de Noronha, C., Yonemoto, W., and Greene, W.C. (2003). HIV-1 Vif blocks the antiviral activity of APOBEC3G by impairing both its translation and intracellular stability. Mol. Cell *12*, 591–601.

Strebel, K. (2007). HIV accessory genes Vif and Vpu. Adv. Pharmacol. *55*, 199–232.

Towers, G.J. (2007). The control of viral infection by tripartite motif proteins and cyclophilin A. Retrovirology *4*, 40.

Uchil, P.D., Quinlan, B.D., Chan, W.T., Luna, J.M., and Mothes, W. (2008). TRIM E3 ligases interfere with early and late stages of the retroviral life cycle. PLoS Pathog. *4*, e16.

Van Damme, N., Goff, D., Katsura, C., Jorgenson, R.L., Mitchell, R., Johnson, M.C., Stephens, E.B., and Guatelli, J. (2008). The interferon-induced protein BST-2 restricts HIV-1 release and is downregulated from the cell surface by the viral Vpu protein. Cell Host Microbe *3*, 245–252.

Vartanian, J.P., Guetard, D., Henry, M., and Wain-Hobson, S. (2008). Evidence for editing of human papillomavirus DNA by APOBEC3 in benign and precancerous lesions. Science *320*, 230–233.

Varthakavi, V., Smith, R.M., Bour, S.P., Strebel, K., and Spearman, P. (2003). Viral protein U counteracts a human host cell restriction that inhibits HIV-1 particle production. Proc. Natl. Acad. Sci. USA *100*, 15154–15159.

Varthakavi, V., Smith, R.M., Martin, K.L., Derdowski, A., Lapierre, L.A., Goldenring, J.R., and Spearman, P. (2006). The pericentriolar recycling endosome plays a key role in Vpu-mediated enhancement of HIV-1 particle release. Traffic *7*, 298–307.

Varthakavi, V., Heimann-Nichols, E., Smith, R.M., Sun, Y., Bram, R.J., Ali, S., Rose, J., Ding, L., and Spearman, P. (2008). Identification of calcium-modulating cyclophilin ligand as a human host restriction to HIV-1 release overcome by Vpu. Nat. Med. *14*, 641–647.

Wolf, D., and Goff, S.P. (2007). TRIM28 mediates primer binding site-targeted silencing of murine leukemia virus in embryonic cells. Cell *131*, 46–57.