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Human Ubc9 Contributes to Production of Fully Infectious Human Immunodeficiency Virus Type 1 Virions[▽]

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Ubc9 was identified as a cellular protein that interacts with the Gag protein of Mason-Pfizer monkey virus. We show here that Ubc9 also interacts with the human immunodeficiency virus type 1 (HIV-1) Gag protein and that their interaction is important for virus replication. Gag was found to colocalize with Ubc9 predominantly at perinuclear puncta. While cells in which Ubc9 expression was suppressed with RNA interference produced normal numbers of virions, these particles were 8- to 10-fold less infectious than those produced in the presence of Ubc9. The nature of this defect was assayed for dependence on Ubc9 during viral assembly, trafficking, and Env incorporation. The Gag-mediated assembly of virus particles and protease-mediated processing of Gag and Gag-Pol were unchanged in the absence of Ubc9. However, the stability of the cell-associated Env glycoprotein was decreased and Env incorporation into released virions was altered. Interestingly, overexpression of the Ubc9 *trans*-dominant-negative mutant C93A, which is a defective E2-SUMO-1 conjugase, suggests that this activity may not be required for interaction with Gag, virion assembly, or infectivity. This finding demonstrates that Ubc9 plays an important role in the production of infectious HIV-1 virions.

Production of infectious human immunodeficiency virus type 1 (HIV-1) particles is a coordinated series of poorly defined events in which the virus takes advantage of cellular components to target its structural proteins and genomic RNAs to common sites on cellular membranes. This colocalization drives the assembly of immature capsids, packaging of the diploid viral genomic RNA, incorporation of viral glycoproteins and virion-associated proteins, and viral budding/release. The major structural component of the immature HIV-1 core is the Gag polyprotein. Gag is synthesized on cytoplasmic ribosomes as a 55-kDa polyprotein, $Pr55_{Gag}$, and is co- or post-translationally myristylated. Expression of Gag alone is sufficient for assembly and release of noninfectious virus-like particles. However, infectivity of HIV-1 can only be attained by inclusion of the receptor-binding Env glycoproteins (gp120 and gp41) and the *pol*-encoded enzymes (protease, reverse transcriptase, and integrase). Protease is essential for the cleavage of the Gag polyprotein into the structural proteins NH_2-MA (p17; the membrane-associated matrix protein), CA (p24; the major capsid protein), SP1 (spacer peptide 1; also known as p2), p17 (NC; the nucleocapsid protein), SP2 (spacer peptide 2; also known as p1), and p6-COOH (17, 73), to form the mature, infectious virion. The cleavage of the precursor polyprotein has been the target of the highly effective protease inhibitor class of antiretrovirals.

Gag-mediated packaging of viral enzymes, structural proteins (e.g., Gag-Pol polyprotein, mature Env glycoproteins, Vpu, Vpr, and Vif), the vRNAs, and cellular components (e.g., cyclophilin A [16] and lysyl-tRNA) is necessary for the production of infectious particles (17, 18, 73). Since these molecules are synthesized in different cellular compartments, it is imperative that cellular pathways traffic these components to common assembly sites on infected cell membranes. Cell fractionation, immunohistochemistry, and electron and fluorescence microscopy studies have shown that Gag assembles and buds almost exclusively from the plasma membrane in primary $CD4^+$ T cells and in most cell lines, such as HeLa, 293, and COS-1 cells (25, 27, 51, 70). However, a significant but minor portion of Pr55 in T cells can also be found associated with late endosomes (LE) (20). These observations led to the postulation that most $Pr55_{Gag}$ buds primarily from the plasma membrane, but a portion of the polyprotein may fail to bud and may cycle off the plasma membrane into the LE (23). Alternatively, it has been suggested that Gag is initially targeted to the LE/multivesicular body compartments and is subsequently transported to the plasma membrane (20, 48, 55, 56, 65). Consistent with the latter hypothesis, further studies have shown that Gag colocalizes with LE/multivesicular body marker proteins in infected/transfected cells and is packaged into released virions (35, 54). However, follow-up studies have shown that the membranes of these intracellular structures are contiguous with the plasma membrane (12, 31, 72), and HIV-1 may utilize these tetraspanin-enriched microdomains as assembly sites (12, 29). The current model is that HIV-1 assembles and buds from specialized regions of the plasma membrane in most, if not all, cell types.

The intracellular trafficking pathways of the viral structural proteins must converge at common sites to allow the requisite assembly interactions to occur. However, the intracellular locations of these interactions and the temporal or spatial reg-

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ulation of these events are not well defined. Gag and Gag-Pol proteins are transported through the cytoplasm to cellular membranes by poorly understood processes that likely involve components of the cellular cytoskeletal system. The viral glycoproteins are synthesized on the rough endoplasmic reticulum (ER) and are cotranslationally translocated into the ER lumen as gp160 precursor proteins. The gp160 molecules are heavily N- and O-linked glycosylated concomitant with trimerization prior to being transported to the Golgi apparatus for oligosaccharide maturation. During transport from the *cis*-medial Golgi to the cell surface, gp160 is likely cleaved by furin in the medial *trans*-Golgi (22, 46), yielding the mature surface (gp120) and transmembrane (gp41) glycoproteins. However, other proteases may also be involved (28, 49, 75).

It is hypothesized that Env trimers interact with Gag at budding sites, yet biochemical and genetic evidence suggests that the initial Gag-Env interactions, whether direct or indirect, occur prior to their arrival at the final assembly sites (41, 42, 61, 64). In fact, Tip47, a tail-interacting protein involved in retrograde transport of cellular factors from endosomes to the *trans*-Golgi network (TGN), was found to be required for Env packaging. This model suggests that during retrograde transport to the TGN, Env comes into contact with Gag on endosomes, with Tip47 directly facilitating Gag-Env interaction. This complex is then targeted to the final assembly site on the plasma membrane (43).

Coordinated trafficking of Gag, Gag-Pol, and Env requires interactions with cellular proteins. A number of host factors, such as AP-1 (7), AP-2 (5), AP-3 (15), Rab9 (47), POSH (2), $PI(4,5)P_2$ (50), Tip47 (43), SOCS1 (58), Arf proteins (30), and KIF4 (44), have been found to interact directly with HIV-1 structural proteins, promoting proper intracellular trafficking and driving virion assembly. Ubc9, an E2 SUMO-conjugating enzyme (63), was found to interact with several different retroviral proteins, but its function during virus replication is unclear. Ubc9 was shown to interact with the full-length Gag protein of Mason-Pfizer monkey virus (M-PMV) near the nuclear membrane, suggestive of a possible function for Ubc9 during virus assembly (71). In contrast, Ubc9 was subsequently shown to interact with and SUMOylate the CA protein of Moloney murine leukemia virus (MLV) (76). Unlike the observations made with M-PMV, an interaction between the full-length MLV Gag protein and Ubc9 was not observed in the yeast two-hybrid assay. Biochemical and genetic analyses suggested that the MLV CA-Ubc9 interaction is required during the early stages of replication, post-reverse transcription.

Finally, Ubc9 was shown to interact with the HIV-1 $p6^{gag}$ protein in yeast and to SUMOylate a p6-green fluorescent protein (GFP) fusion protein in mammalian cells. Overexpression of SUMO-1 resulted in the packaging of free SUMO-1 into HIV-1 virions and was shown to cause a fivefold decrease in the infectivity of these virions compared to virions produced in control cells. It was found that the defect in infectivity of wild-type (wt) HIV-1 virions produced in the presence of overexpressed SUMO-1 occurred during a postentry step (21), which suggests that SUMOylation of p6 negatively regulates HIV-1 infectivity during an early infectious event. Interestingly, mutation of the SUMOylation site, in the context of an infectious provirus, had no apparent effect on virus replication in normal cells. However, unlike wt HIV-1, the SUMOylation mutant did not exhibit a defect in virion infectivity when SUMO-1 was overexpressed in producer cells. Thus, the role of SUMOylation in p6 function is still not clear.

Recently, it was reported that GagGFP fusion protein colocalizes with myc-Ubc9 at Gag perinuclear clusters (GPCs) in the absence of KIF4 (44), a kinesin family motor protein. Blocking KIF4 function resulted in Gag accumulation at GPCs, inhibiting Gag trafficking, stability, and subsequent release. However, neither an interaction between Ubc9 and full-length Pr55 nor SUMOylation of Pr55 by Ubc9 during assembly was examined in the previous reports. In this study, we further investigated the role of Ubc9 during HIV-1 replication. We demonstrated that (i) Ubc9 interacts with Pr55; (ii) Ubc9 and C93A Ubc9, a *trans*-dominant negative mutant (68), colocalize with Gag at perinuclear sites; (iii) Ubc9 is crucial during the late stages of replication to produce infectious particles; and (iv) the E2 SUMO conjugation activity of Ubc9 does not appear to be a prerequisite for its effect during the late stages of the HIV-1 life cycle.

MATERIALS AND METHODS

Cell lines and bacterial strains. TZM-bl indicator cells were obtained from the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: TZM-bl from John C. Kappes, Xiaoyun Wu, and Tranzyme Inc. The HeLa and 293T cells were obtained from the American Type Culture Collection. *Escherichia coli* BL21 (DE3) cells were used for expression of glutathione *S*-transferase (GST) and GST-Ubc9 (71).

Plasmids. The infectious HIV-1 proviral clone pNL4-3 and the noninfectious clone pNL4-3-ΔE-EGFP were obtained from the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: pNL4-3 from Malcolm Martin (1) and pNL4-3-ΔE-EGFP (catalog no. 11100) from Haili Zhang, Yan Zhou, and Robert Siliciano (78). The pLTR Env_{NL4-3} construct encoding HIV-1 envelope was created by introducing an EcoRI site into pNL4-3 at position 823. The 4,920-bp EcoRI (nucleotides 823 and 5743) fragment was removed, and the remaining vector was religated. The plasmids pCMVgagpol-RRE-R and pCMVrev, encoding HIV-1 Gag and Rev, respectively, were a kind gift from David Rekosh (University of Virginia, Charlottesville) (37, 67). The in vitro Gag expression plasmids pTM/55BAM, pGPG1-39G1, pGPG1-15G1, pGPG1-41G1, pGPG1-17G1, and pGPG1-24G1 were a kind gift from Paul Spearman (Emory University, Atlanta, GA) (66). The plasmid pHyg-VSV-G, encoding vesicular stomatitis virus G protein, was a kind gift from Asit Pattnaik (University of Nebraska—Lincoln). Plasmids pET41c+ (Novagen), pET Ubc9, pCMV-Tag3A, and pCMV Myc-Ubc9 have been previously described (71). Myc-C93A was created by PCR-based site directed mutagenesis of Myc-Ubc9 by mutating nucleotides 276 and 277 to cytosine and guanine, respectively, in the Ubc9 open reading frame. The plasmid pEGFP-SUMO-1, encoding an enhanced-GFP (EGFP)–SUMO-1 fusion protein, was a kind gift from Yongsok Kim (33).

RNA interference (RNAi) and DNA transfections. Synthetic double-stranded small interfering RNAs (siRNAs) with 3′ dTdT overhangs, corresponding to nucleotides 86 to 104 of the Ubc9 open reading frame, were purchased from Dharmacon and are similar to those previously described (39). Fluorescent, nonsilencing 21-bp double-stranded RNA (Qiagen) was used as a control RNA (Ctr. RNA). 293T cells were transfected twice with RNAs, at 0 and 24 h, using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's protocol. After an additional 24 h, the cells were transfected with either plasmid pNL4-3, pLTR Env_{NL4-3}, pNL4-3-ΔEnv-EGFP, or pNL4-3-ΔE-EGFP with pHyg-VSV-G. Plasmids pCMVgagpol-RRE-R and pCMVrev were cotransfected into HeLa cells using Fugene 6 (Roche) according to the manufacturer's suggestions.

GST pull-down assays. pTM/55BAM, pGPG1-39G1, pGPG1-15G1, pGPG1-41G1, pGPG1-17G1, and pGPG1-24G1 were used to express [^{35}S]methionine-cysteine-radiolabeled wt Gag and Gag deletion mutants using the TNT Coupled Reticulocyte Lysate System (Promega). GST-Ubc9 (pET Ubc9) and GST (pET41c+) were expressed in and purified from *E. coli* as previously described. Radiolabeled Gag or Gag deletion mutants were incubated with equimolar concentrations of GST or GST-Ubc9 and immobilized on glutathione beads, and pull-down assays were carried out as previously described (71).

Viral-infectivity assays. Virus released into the culture media from transfected cells was quantified by determining the amount of released p24 by immunoblotting. TZM-bl indicator cells were infected with equivalent amounts of viruses (normalized to the p24 content). The number of infected cells in this infectivity assay was determined by staining for β-galactosidase activity at 36 h postinfection as previously described (34).

Antibodies. Anti-Ubc9 (H-81) rabbit polyclonal antibodies (PAb), anti-actin goat PAb, anti-cyclophilin A PAb, HIV-1 anti-gp41 (10E9) monoclonal antibodies (MAb), and horseradish peroxidase (HRP)-conjugated chicken anti-goat PAb were purchased from Santa Cruz Biotechnology, Inc. The following rabbit, goat, and mouse anti-HIV-1 antibodies were obtained from the NIH AIDS Research and Reference Reagent Program: anti-p24 MAb 24-2, gp41 MAb 2F5, HIV-1_{sf2} p24 PAb, HIV-1 gp160/120 goat PAb H3T, HIV-1_{HXB2} Vif PAb, HIV-1_{NL4-3} Vpu PAb, and Vpr PAb. Anti-SUMO-1 no. 12783 rabbit PAb, a kind gift from Van Wilson (Texas A&M University System Health Science Center, College Station), were previously described (57). HRP-conjugated goat anti-rabbit and HRP-conjugated sheep anti-human antibodies were purchased from Amersham Pharmacia Biotech. Cy5-conjugated donkey anti-mouse and Cy2-conjugated donkey anti-rabbit sera were purchased from Jackson Immunoresearch Laboratories. Pooled AIDS patient sera and pooled normal human sera were obtained from a patient cohort.

Immunoblotting analysis. Media from transfected cells were harvested, and virions were purified from the media by pelleting them through a 20% sucrose cushion (6). The pelleted virions and cells were solubilized directly in 2× protein sample buffer (PSB), separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and analyzed by immunoblotting. For immunoblot detection of SUMOylated proteins, transfected cells were lysed as previously described (4) with SUMO lysis buffer containing 20 mM iodoacetamide and *N*-ethylmaleimide. The lysates were separated by SDS-PAGE, transferred to a polyvinylidene difluoride membrane (GE Healthcare), and analyzed by immunoblotting.

vRNA extraction and real-time PCR. Viral RNA (vRNA) was extracted from equal numbers of virions (normalized by p24 content) and reverse transcribed (6). Viral cDNA was quantified by real-time PCR (iCyclerIQ; Bio-Rad) using previously described primers (10).

Cell viability assay. The viability of Ubc9 siRNA-transfected cells and control cells was assayed using BD ApoAlert Annexin V and an Apo 2.7-PE kit (BD Biosciences) according to the manufacturer's protocol. Annexin-EGFP V staining and propidium iodide uptake were measured using a BD FACSCalibur (BD Biosciences).

Confocal microscopy. Transfected 293T cells were cultured on sterile coverslips treated with poly-L-lysine in 35-mm culture dishes. The cells were fixed at room temperature in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 20 min and permeabilized with 0.2% Triton X-100 in PBS for 5 min. The fixed cells were processed and stained as previously described (6). The coverslips were mounted on slides and analyzed by confocal microscopy (Olympus FV500 with an upright BX Olympus fluorescence microscope). Proteins in transfected cells were detected using the following antibodies: Pr55, anti-p24 MAb, Env, anti-gp41 MAb 10E9, Ubc9, and anti-Ubc9 PAbs. Fluorescent Cy5-conjugated donkey anti-mouse and Cy2-conjugated donkey anti-rabbit antibodies were used as secondary antibodies. Anti-Ubc9 antibodies were not able to detect endogenous Ubc9 in this assay. Consequently, Cy2 background antibody staining and any potential low-level endogenous Ubc9 staining signals were reduced to undetectable levels; thus, the fluorescent signals detected were due to exogenous protein expression only. Z sections (0.3 μm) were analyzed using Flowview imaging software (Olympus).

Metabolic labeling and immunoprecipitation. Transfected 293T cells were pulse-labeled for 45 min with 300 μCi [^{35}S]methionine/cysteine (>1,000 Ci/mmol; NEN). The labeling media were removed, and the cells were chased for 2 and 4 h in normal growth media. The cell culture supernatants were then collected, clarified by centrifugation (13,800 × *g* for 1 min), and adjusted to 1× lysis buffer (60). The cells were lysed with 1× lysis buffer containing protease inhibitors (Roche) and centrifuged to remove cellular debris. Viral proteins were immunoprecipitated with pooled patient sera, separated by SDS-PAGE, and visualized by fluorography. For coimmunoprecipitation experiments, transfected HeLa cells were lysed with GTN buffer containing 0.1% bovine serum albumin (71). The cell lysates were centrifuged to remove cellular debris, Ubc9-Gag complexes were immunoprecipitated with anti-Ubc9 antibodies that recognize both endogenous Ubc9 and Myc-Ubc9, and the complexes were detected by immunoblotting using anti-p24 sera.

Quantitation of gp160 processing efficiency. Autoradiograms were scanned using a Fluor-S system. The accompanying Discovery Series Quantity One software (Bio-Rad) was used to quantitate the intensities of the bands corresponding to gp160 and gp120 from the scanned images. The molar equivalence of each protein was calculated by dividing the band intensity by the number of methionines for each protein (gp160 and gp120 contain 14 and 9 methionines, respectively). To calculate the percentage of gp160 cleaved into gp120 in cells expressing Env only, the molar equivalence of gp120 was divided by the total molar equivalence of Env (gp120 and gp160).

RESULTS

HIV-1 Gag interacts with hUbc9. While we have shown that Ubc9 interacts with the full-length Gag protein of M-PMV, others have not detected an interaction with the full-length Gag proteins from other retroviruses. However, a Gag cleavage product, p6, was identified as a Ubc9 binding partner and target for SUMOylation (21). Furthermore, recent work has shown that HIV-1 GagGFP fusion protein is targeted to a structure containing Ubc9 and SUMO-1 early in the assembly pathway, suggesting that Ubc9 may play a role during the assembly process (44). To test if an interaction between Ubc9 and HIV-1 Pr55 could be detected in vitro, Pr55 was translated in vitro in the presence of [^{35}S]methionine. GST-tagged Ubc9 was expressed in *E. coli* and purified using glutathione-agarose beads. The GST-Ubc9-bound beads were mixed with in vitro-translated Pr55. The bound proteins were eluted and examined by SDS-PAGE and fluorography (Fig. 1a). While GST control beads showed no detectable binding to Pr55, the GST-Ubc9 beads efficiently bound Pr55 under conditions previously used to detect Ubc9–M-PMV Gag interactions. The 35-kDa band that was pulled down with GST-Ubc9 is a peptide produced during the in vitro transcription reaction from the use of an internal ATG as the translation start site. This peptide could contain the Gag/Ubc9 binding site. GST-Ubc9 pull-down assays using a panel of Gag deletion mutants (Fig. 1b) showed that the Ubc9 binding site on Pr55 is located within the carboxy-terminal NC-p2-p6 region, since only clones pGPG1-39G1 and pGPG1-15G1 retained the ability to be pulled down by GST-Ubc9 (Fig. 1c). These results were consistent with previous observations that the p6 domain of Pr55 contains a Ubc9 binding site (21) and demonstrated that the MA-CA-p1 regions of Pr55 do not contain any additional major Ubc9 binding sites.

Coimmunoprecipitations confirmed that Ubc9 and Gag interact in vivo (Fig. 2a). pNL4-3 or Pr55 was expressed in HeLa cells alone or with Myc-tagged Ubc9. At 48 h posttransfection, the cells were lysed using the same buffer used in the GST pull downs and incubated with either polyclonal anti-Ubc9 antibodies or preimmune rabbit sera. Immunoblots using anti-p24 sera indicated that while the control preimmune sera did not precipitate Pr55, anti-Ubc9 antibodies coimmunoprecipitated Pr55. The overexpression of Myc-tagged Ubc9 only increased the amount of Pr55 pulled down by about 30%. The small increase in the amount of Gag pulled down when Ubc9 was overexpressed was not unexpected, as Ubc9 interactions with target proteins were shown to be transient and weak (38).

To further substantiate Ubc9-Pr55 interactions in cells, immunofluorescence assays were utilized to examine Ubc9 and Gag subcellular localization. 293T cells were transfected with either Myc-Ubc9 expression plasmid alone (Fig. 2b to d) or cotransfected with pNL4-3 and Myc-Ubc9 (Fig. 2e to j). The cells were processed 48 h posttransfection and examined by confocal microscopy. Myc-Ubc9 staining occurred in the nucleus, throughout the cytoplasm, in areas near the plasma

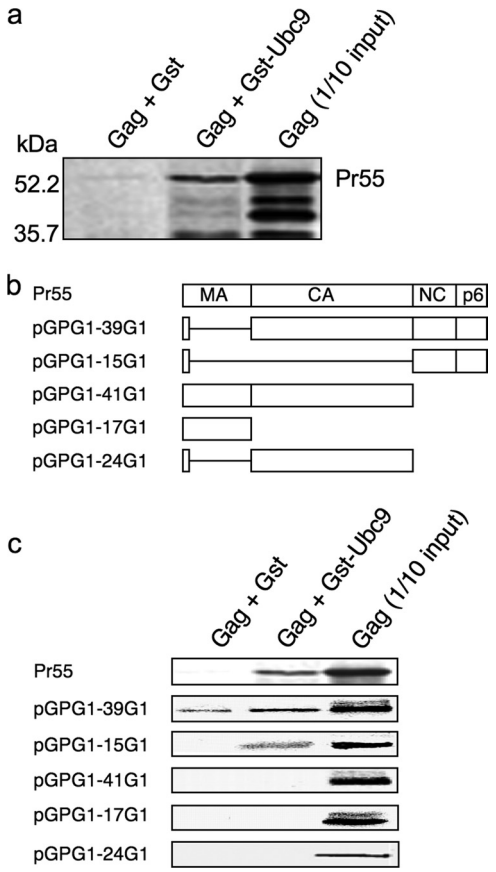

FIG. 1. HIV-1 Gag proteins interact with Ubc9 in vitro. (a) GST-Ubc9 pull-down assay. [^{35}S]methionine-labeled in vitro-translated Pr55 was incubated in GTN buffer with GST-bound glutathione beads or GST-Ubc9-bound beads. As a loading control, 1/10 of the amount of Pr55 added to the GST pull-down reaction mixtures was loaded directly. Bound proteins were eluted with PSB and separated by SDS-PAGE. The radiolabeled proteins were visualized by phosphorimaging. (b) Mapping the Ubc9 binding domain within HIV-1 Gag. (c) GST-Ubc9 pull-down assays using wt Gag and the Gag deletion mutants were done as described above.

membrane (Fig. 2b to d, e, and h), and as distinct foci juxtaposed to the nuclear membrane (Fig. 2b to d, e, and h). In cells cotransfected with Myc-Ubc9 and pNL4-3 (Fig. 2e to j), HIV-1 Gag stained the cytoplasm and plasma membrane and at perinuclear foci (Fig. 2f). HIV-1 Gag and Ubc9 colocalized in HIV-1-expressing cells (Fig. 2g) in a similar pattern that was observed with M-PMV Gag and Ubc9 in infected cells (71) and is similar to what was observed recently for HIV-1 GagGFP fusion protein (44). Gag was also observed to colocalize with Ubc9 in the cytoplasm near the plasma membrane and at perinuclear foci (Fig. 2g). In an effort to identify the Gag-Ubc9 colocalization site and to determine whether Ubc9 and Env also colocalize, we stained for both Env and Ubc9. Env and Ubc9 did not seem to colocalize (Fig. 2h to j), and certainly not at the same punctate perinuclear site observed between Gag and Ubc9. Furthermore, these results suggest that the Ubc9/Gag foci observed in this study are not in the ER, Golgi apparatus, or post-Golgi vesicles, all cellular organelles that Env is known to occupy. These results are similar to those reported by Martinez et al., where they described a similar subcellular site containing Gag, Ubc9, and SUMO-1 (44).

Ubc9 is important for the production of infectious HIV-1 particles. Having determined that Ubc9 interacts with the full-length HIV-1 Gag protein, we sought to further elucidate the role of Ubc9 in the production of replication-competent HIV-1 particles by ascertaining whether infectious virions could be produced in the absence of Ubc9 expression. To achieve this, 293T cells were transfected twice with either a nonsilencing Ctr. RNA or a Ubc9-specific silencing RNA (Ubc9 RNAi) at 24-h intervals, followed by transfection with wt HIV-1 proviral DNA (pNL4-3) after an additional 24 h. The effects of Ubc9 RNAi transfection on Ubc9 expression, cell viability, virus production, and viral infectivity were assayed 72 h after the initial RNA transfection. As shown in Fig. 3a, Ubc9 expression was reduced to almost undetectable levels in cells transfected with Ubc9 RNAi compared to cells transfected with the Ctr. RNA. The levels of the controls, p53 and actin, remained constant in all samples, demonstrating the specificity of Ubc9 silencing. Because Ubc9 is required to sustain cellular replication (24), we examined cell viabilities at 72 h posttransfection, when media containing virions were collected. Flow cytometry quantifying propidium iodide incorporation, Annexin V staining, and trypan blue exclusion (data not shown) assays demonstrated that greater than 97% of control RNA- and Ubc9 RNAi-transfected cells were viable when the virions were harvested (Fig. 3b).

To examine whether Ubc9 is important during virus assembly and release, cell-free virions released into culture supernatants from transfected cells were pelleted, lysed, and analyzed by Western blotting. We found that Ubc9 expression is not required for virus assembly, budding, or Pr55 processing, since equivalent amounts of p24 were released from cells as pelletable virus particles in the presence or absence of Ubc9 expression (Fig. 3c). These virions also contained equivalent amounts of reverse transcriptase (data not shown). The effect of Ubc9 depletion on virion infectivity was analyzed by using equivalent numbers of virions (normalized to p24 content) released from these cells in an infectivity assay using TZM-bl reporter cells to quantify the infectious events relative to virions produced in the presence of Ubc9. Virions released from cells that did not express detectable levels of Ubc9 were on average 8- to 10-fold less infectious than those virions released from cells expressing normal levels of Ubc9 (Fig. 3d). The average viral titers for cells transfected with no RNA, Ctr. RNA, and Ubc9 RNAi were 3,750, 4,083, and 367, respectively. These results are the first to demonstrate that while Ubc9 has no effect on Gag synthesis, processing, or assembly into virions, its expression in the producer cells is important for the generation of infectious HIV-1 virions.

Ubc9 is important for the correct production and packaging of viral components. We initially anticipated that Ubc9 must be packaged into HIV-1 virions to achieve full infectivity. However, this scenario appears unlikely, since we and others (9) were unable to detect Ubc9 or SUMO-1 in HIV-1 particles released from cells even when myc-Ubc9 was overexpressed in the producer cells (data not shown). Our results (Fig. 3c) demonstrated that the interaction between Gag and Ubc9 is not required for Gag release and processing but is important for infectivity (Fig. 3d). Therefore, Ubc9 must influence Gag-

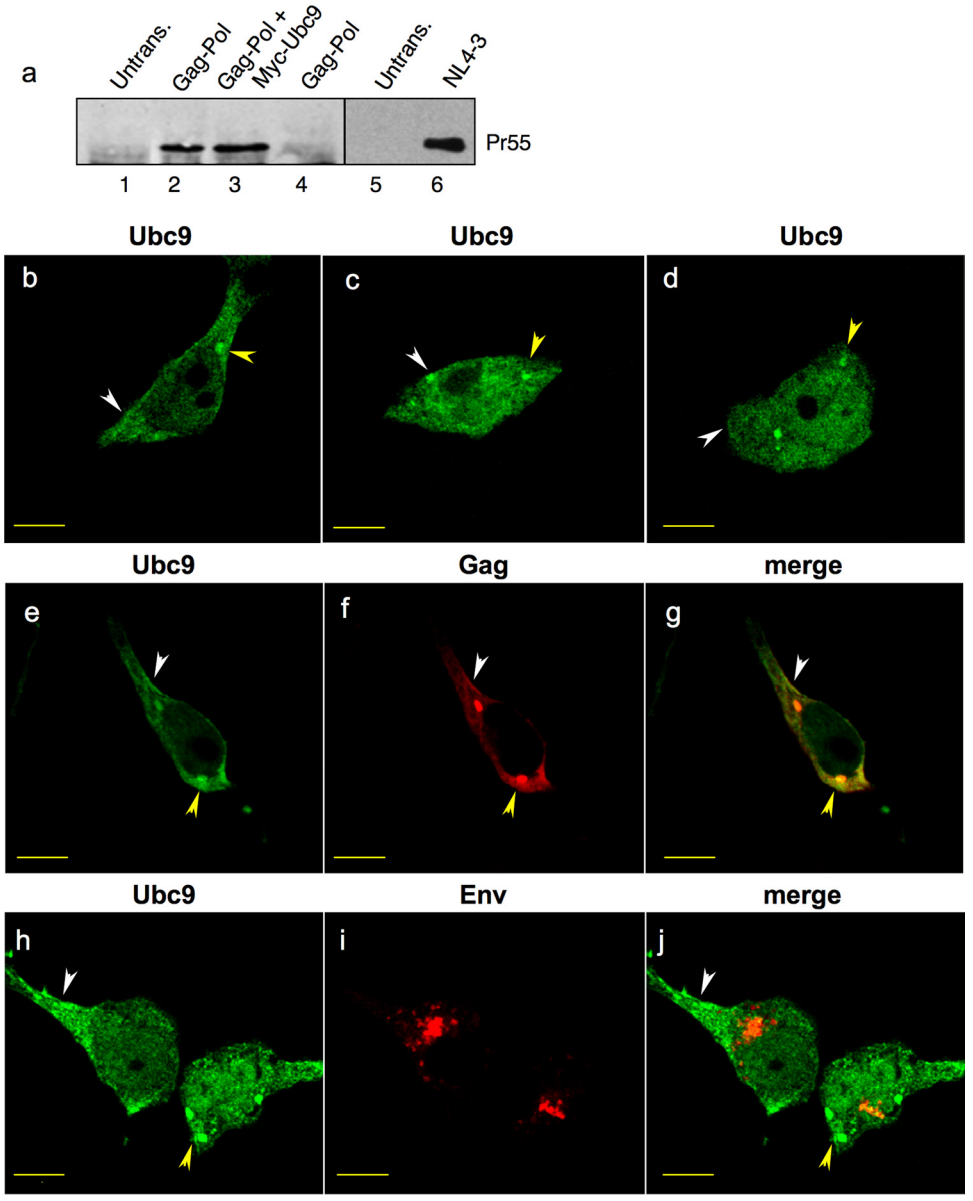

FIG. 2. HIV-1 Gag proteins interact with Ubc9 in vivo. (a) Coimmunoprecipitation of endogenous Ubc9 and HIV-1 Gag. HeLa cells were either mock transfected (Untrans.) (lanes 1 and 5); cotransfected with pCMVgagpol-RRE-R and pCMVrev (lane 2 and lane 4); cotransfected with pCMVgagpol-RRE-R, pCMVrev, and pCMV Myc-Ubc9 (lane 3); or transfected with pNL4-3 (lane 6). The cells were lysed with GTN buffer and immunoprecipitated with anti-Ubc9 (lanes 1, 2, 3, 5, and 6) or preimmune sera (lane 4), and analyzed by immunoblotting using anti-p24 antibodies. (b to j) Gag and Ubc9 colocalize at distinct perinuclear puncta and areas near the plasma membrane. 293T cells were transfected with Myc-Ubc9 (b to d) or cotransfected with pNL4-3 and Myc-Ubc9 expression plasmid (e to j). The cells were fixed and processed for immunofluorescence with anti-Ubc9 PAb (b to d), anti-p24 MAb and anti-Ubc9 PAb (e to g), or anti-Ubc9 PAb and 10E9 anti-Env MAb (h to j). Cy5-conjugated donkey anti-mouse and Cy2-conjugated donkey anti-rabbit antibodies were used as secondary antibodies. Colocalization was examined by confocal microscopy using 0.3-μm optical sections. Representative medial sections are shown. The yellow arrowheads indicate the perinuclear sites where Ubc9 and Gag should colocalize. The white arrowheads indicate areas where Ubc9 and Gag should colocalize near the plasma membrane. Scale bars, 10 μm.

mediated biosynthesis, the packaging of other viral components, or the inclusion of cell-derived viral inhibitors. The deficiency in assembly of released virions was identified by comparing the composition of replication-defective virions harvested from Ubc9 knockdown cells to that of fully infectious HIV-1 from normal and control RNA-transfected cells.

As shown, Ubc9 expression was not required for packaging of the vRNA (Fig. 4a); the HIV-1 accessory proteins Vpr, Vpu, and Vif; or the cellular protein cyclophilin A (Fig. 4b). In contrast, virions produced in the absence of Ubc9 expression contained significantly smaller amounts of Env glycoproteins (gp120, and gp41) than virions produced from cells transfected with pNL4-3 alone or with pNL4-3 and control RNA (Fig. 4c). Env production was assayed in these producer cells, and it was

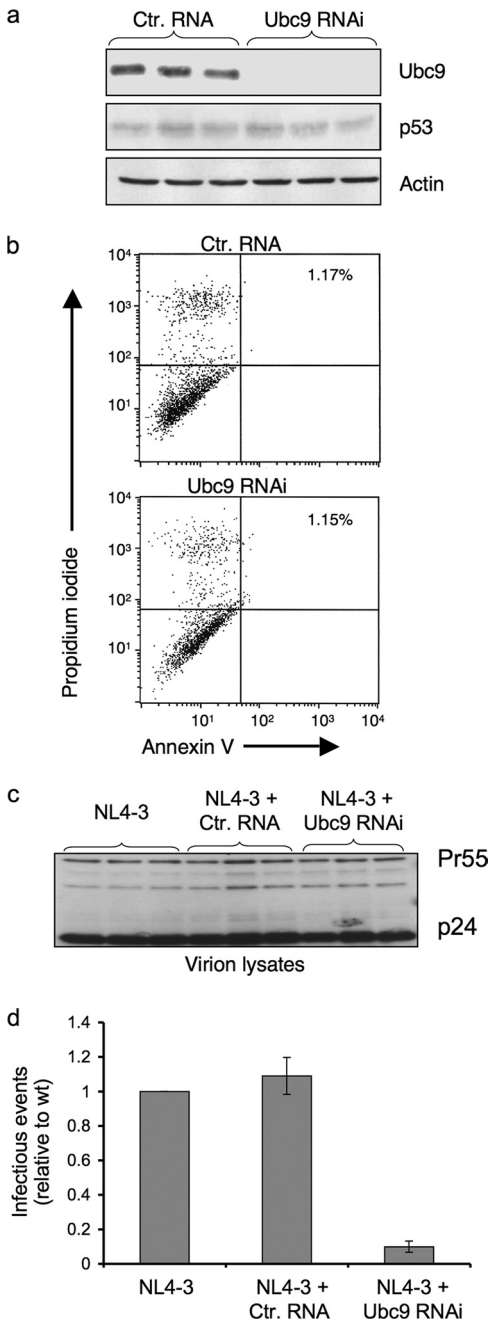

FIG. 3. Ubc9 is important for the production of infectious HIV-1 virions. (a) Ubc9 expression can be suppressed by RNA silencing. 293T cells were transfected with either Ctr. RNA or Ubc9 siRNA. A second transfection with the RNAs was performed 24 h later. The cells were lysed in PSB and separated by SDS-PAGE, and protein expression levels were analyzed by immunoblotting using antibodies against Ubc9, actin, and p53 72 h after the initial siRNA transfection. (b) Suppression of Ubc9 does not affect cell viability. The viability of Ctr. RNA- and/or Ubc9 RNAi-transfected 293T cells was analyzed using the BD ApoAlert Annexin V and Apo 2.7-PE kit following the manufacturer's suggested protocol. The percentages of cells undergoing apoptosis were measured by flow cytometry. (c) Ubc9 is not required for budding. 293T cells were either left untreated or transfected twice with Ubc9 siRNA or Ctr. RNA. Wt HIV-1 proviral DNA (pNL4-3) was transfected 24 h following the second siRNA transfection. Twenty-four hours later (72 h after the initial siRNA transfection), the culture medium was collected and clarified. Virions in the culture medium were pelleted through a 20% sucrose cushion and lysed with PSB. Equal volumes of the lysates were separated by SDS-PAGE, and p24 levels were measured by immunoblotting using anti-p24 antibodies. (d) Ubc9 in virus-producing cell is required for HIV-1 infectivity. An amount of virus normalized to pelletable p24 present in the medium from transfected 293T cells was used to infect TZM-bl indicator cells for 36 h. The numbers of infectious events were analyzed by counting the cells that were positive for X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) staining. The error bars indicate standard deviations.

found that cells transfected with pNL4-3 and Ubc9 RNAi contained similar amounts of the Env precursor protein gp160 but exhibited a decrease in gp120 and gp41 levels compared to those that were transfected with pNL4-3 alone or with pNL4-3 and Ctr. RNA (Fig. 4d).

The changes observed in gp120 expression and packaging were unexpected and suggested that the decrease in virion infection may be due to the inability of the virions to attach to and enter a susceptible cell; however, the SUMOylation pathway has also been implicated during early infectious events of HIV-1 (21) and MLV (76). Gurer et al. had previously shown that overexpression of SUMO-1 in producer cells leads to a decrease in HIV-1 virion infectivity, which was attributed to a defect during an early step of reverse transcription of the viral genome. Similarly, Yueh et al. reported that point mutations of lysines (K202 and K220) that block the SUMOylation of the capsid protein of MLV also resulted in a defect at an early infectious event, albeit at a post-reverse-transcription step.

To examine the possibility that the decrease in virion infectivity could be attributed to defects that occurred during postentry events, we assayed the ability of vesicular stomatitis virus G protein-pseudotyped HIV-1 cores to complete early infection events and nascent viral-gene expression. These experiments allowed us to examine early events independently of any potential defects in entry caused by the decreased Env packaging in the absence of Ubc9 expression. Virion infectivity was carried out as before using TZM-bl indicator cells. As shown in Fig. 4e, the decreased expression of Ubc9 by RNAi had no affect on the infectivity of the pseudotyped HIV-1 cores. These data indicated that the defect observed with virions produced in the absence of Ubc9 expression was not due to a defect during early postentry events but was likely the result of the virions' inability to enter a susceptible cell as a result of the decreased amount of Env packaged.

Ubc9 contributes to viral glycoprotein stability in the presence of Gag expression. Immunoblot data that examined viral proteins at steady-state levels showed a significant decrease in the amount of gp120/gp41 in the transfected cells and in the amount of Env packaged into the virions. The decrease in gp120 in the producer cells suggested that this could be due to a defect in gp160 maturation, a decrease in the stability of gp120, or a lower number of mature virions being endocytosed in the absence of Ubc9 expression. A defect in gp160 maturation was less likely, as immunoblot data (Fig. 4d) did not show the increase in gp160 expression levels at steady state, which was expected if gp160 was not undergoing cleavage during maturation. Therefore, pulse-chase experiments were carried out to further examine these possibilities and to access Env

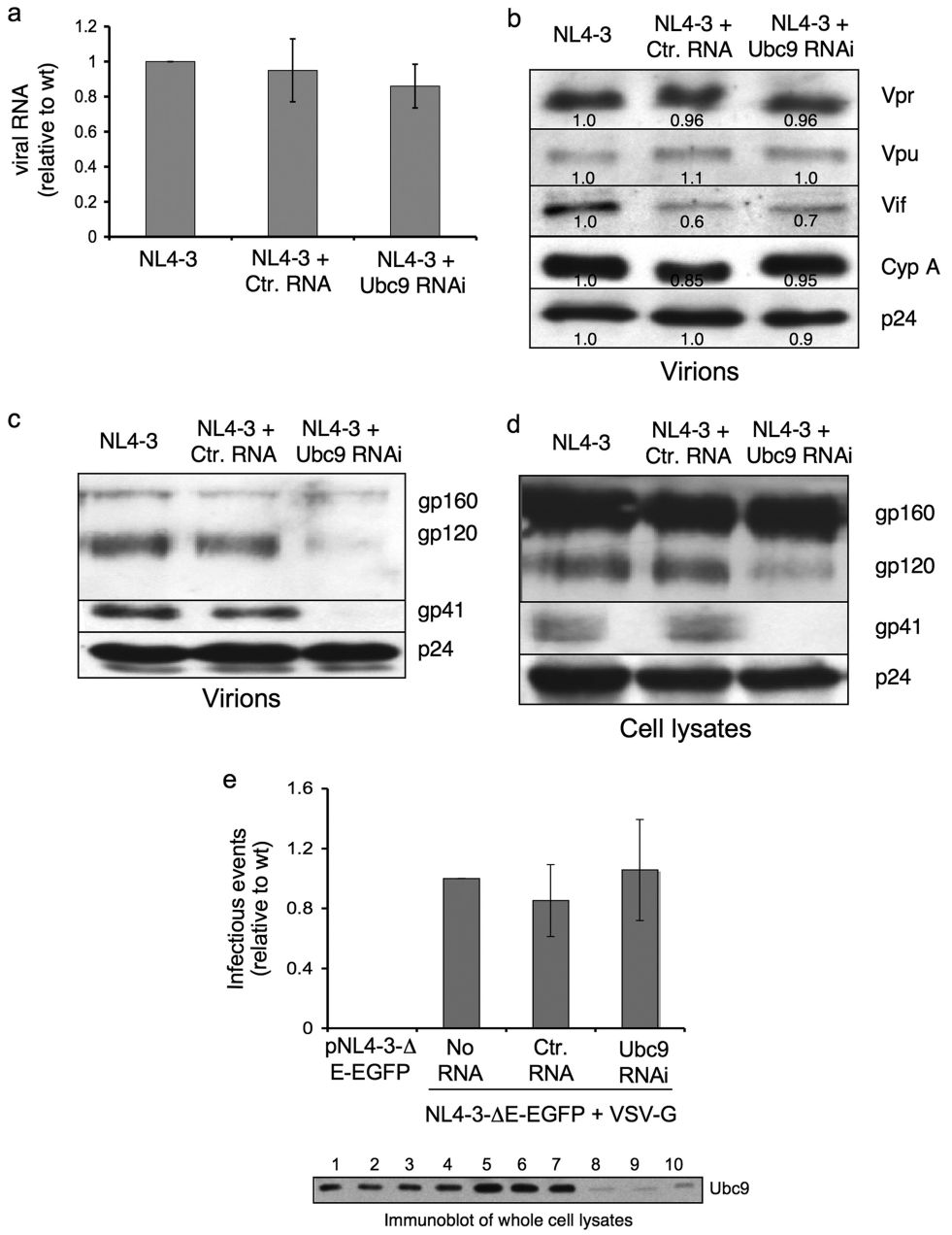

FIG. 4. Biochemical composition of viral particles. The relative levels of vRNAs and proteins packaged into equivalent numbers of defective and infectious virions (normalized to the p24 content) were analyzed. (a) VRNA packaging. RNA from pelleted virions was extracted, reverse transcribed using oligo(dT) 12-18, and quantified by real-time PCR using vRNA-specific primers. Real-time PCR data from three independent experiments is shown as the amount of vRNA packaged into virions relative to that of vRNA packaged into virions produced from cells transfected with pNL4-3 only. The error bars indicate standard deviations. (b) Packaging of cellular and HIV-1 regulatory proteins. The levels of cellular (cyclophilin A [Cyp A]) and viral (Vif, Vpr, and Vpu) regulatory proteins packaged into virions were analyzed by immunoblotting. The bands were quantified using Discovery Series Quantity One software. (c) Glycoprotein packaging into virions. Envelope packaging into virions was analyzed by immunoblotting using polyclonal anti-HIV-1 and monoclonal anti-gp41 antibodies. (d) Glycoprotein production in cell lysates. Envelope production inside transfected cells was analyzed by immunoblotting using polyclonal anti-HIV-1 and monoclonal anti-gp41 antibodies. (e) Decreases in virion infectivity are not due to defects in early postentry events. Cells were transfected with RNAs as in previous experiments, followed by DNA transfections with pNL4-3-ΔE-EGFP and pHyg-VSV-G or pNL4-3 ΔEnv alone. Media containing pseudotyped virions were harvested and clarified 24 h after DNA transfections. Virion infectivity assays were carried out as before using TZM-bl target cells. Ubc9 expression in cell lysates. No RNA (lanes 1 to 4), control RNA (lanes 5 to 7), Ubc9 RNAi (lanes 8 to 10).

stability in the absence of Ubc9 expression. Equivalent amounts of Env and Gag proteins were synthesized in all samples (Fig. 5a). However, a 90% reduction in Ubc9 expression resulted in an approximately twofold decrease in the amount of cell-associated gp120 (Fig. 5a and c). This reduction in cell-associated gp120 was not due to a defect in gp160 processing or an increased release of gp120 into the media (Fig. 5a and b). When pulse-labeled Gag proteins were chased,

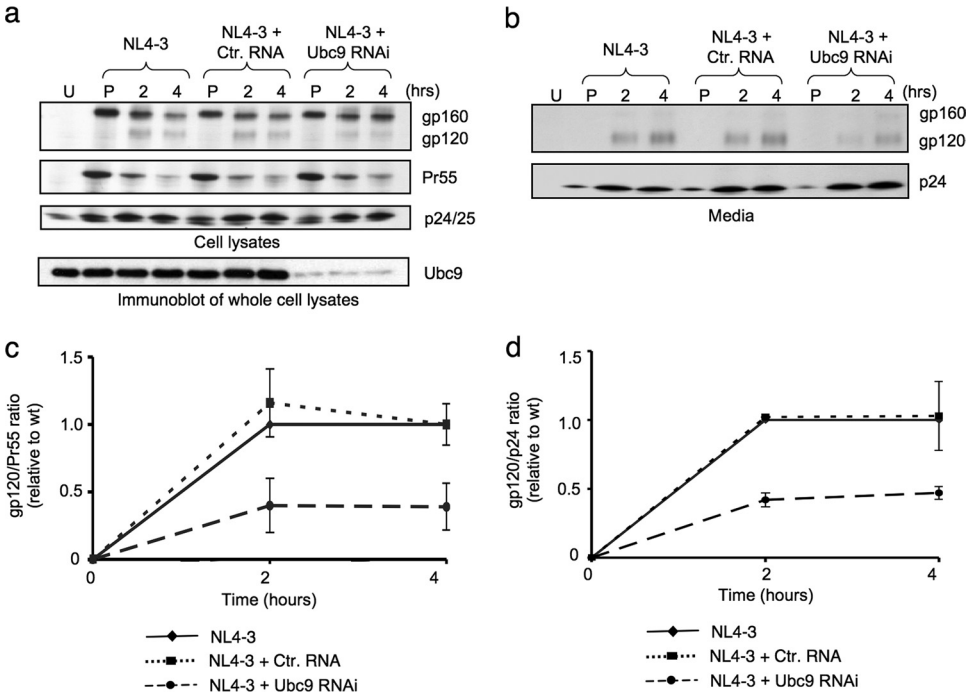

FIG. 5. Gag and Env processing in the absence of Ubc9. (a and b) Viral-protein assembly kinetics. 293T cells were transfected with NL4-3, NL4-3 plus Ctr. RNA, or NL4-3 plus Ubc9 RNAi or left untransfected (U). The cells were pulsed (P) with [^{35}S]methionine for 45 min and then chased for 2 and 4 h. HIV-1 proteins in the cell lysates (a) and the culture supernatants (b) were immunoprecipitated with pooled AIDS patient sera, separated by SDS-PAGE, and detected by fluorography. The viral-protein bands from multiple pulse-chase experiments were quantified and analyzed using Discovery Series Quantity One software. (c) Ratio of cellular gp120/Pr55. (d) Ratio of gp120/p24 released into the media. The error bars indicate standard deviations.

the levels of cell-associated Pr55 decreased, as virus-associated p24 increased at the same rates regardless of the Ubc9 expression levels. The amount of cell-associated p25/p24 did not change relative to the control cells, indicating that the decrease in cell-associated gp120 was not due to decreased endocytosis of mature virions.

The amount of gp120 packaged into particles released from Ubc9 RNAi-treated cells was also approximately twofold less than that packaged into the particles released from control cells. This is consistent with what was observed in the amounts of cell-associated gp120. The small amount of gp120 that was detected in Ubc9 RNAi-transfected cells and released into the media was probably from cells that were not transfected with the Ubc9 RNAi. These data suggest that Ubc9 plays an important role in determining gp120 levels in the virus-producing cells, either directly or indirectly, resulting in decreased packaging of gp120 into the released virions.

Ubc9 has been reported to interact with a variety of cellular proteins affecting subcellular location, function, and stability (45). Some of these proteins have biosynthetic pathways similar to those of HIV-1 Env and are found in organelles known to be utilized by Env in the late virion assembly pathway (11, 62, 74). Because Ubc9 has been shown to act directly upon cellular glycoproteins utilizing the secretory pathway, pulse-chase experiments that assayed Env stability were done in the absence of Gag expression to rule out the possibility that Ubc9 was directly acting upon Env. This was carried out by using pNL4-3 that was modified to express Env glycoproteins in the absence of Pr55 by deleting the *gag* and *pol* genes. 293T cells were transfected with this construct (long terminal repeat [LTR] Env_{NL4-3}) either alone or with the Ctr. RNA or Ubc9 siRNA. Env-processing kinetics and stability were examined by pulse-chase experiments at 24 h posttransfection. Surprisingly, in the absence of Gag, pretreatment of cells with Ubc9 RNAi had no effect on the amount of gp160 that was synthesized and processed or on the kinetics and efficiency of processing gp160 into gp120 and gp41 (Fig. 6). We concluded from these experiments that Ubc9, in the absence of Gag, does not play a significant role either in gp160 cleavage, in trafficking through the secretory pathway to the TGN, or in gp120 stability. Together, these results demonstrate that Ubc9 functions during the virus assembly process, which is required for full infectivity, and suggest that Ubc9 is involved in the processing and packaging of mature Env glycoproteins.

Ubc9 functions independently of its E2 SUMO conjugase activity. Ubc9 is the only E2 SUMO-conjugating enzyme identified (19), and many of the Ubc9-interacting partners are substrates for SUMOylation. However, there are an increasing number of reports that demonstrate that Ubc9 also functions in a SUMO conjugase-independent manner (3, 8, 32, 36, 53, 59, 69). To determine whether Ubc9 SUMO conjugase activity is involved or required for its interaction with Gag and if it affects viral infectivity, we tested the effect of the overexpression of a *trans*-dominant-negative mutant, C93A, that has no E2 SUMO conjugase activity (68). 293T cells were cotransfected with equal amounts of EGFP–SUMO-1 and pCMV-Tag3A (empty vector) or EGFP–SUMO-1 and Myc-C93A or with pCMV-Tag3A alone to confirm that Myc-C93A acts as a

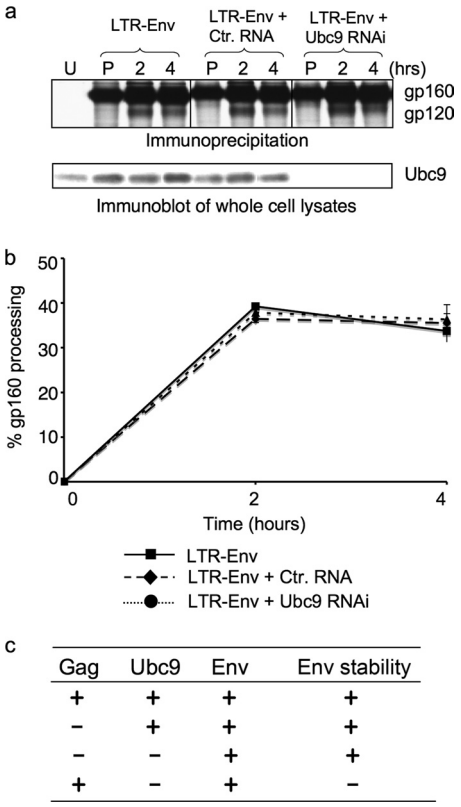

FIG. 6. Env processing in the absence of Gag and Ubc9 expression. (a) Ubc9 does not directly affect Env processing. 293T cells were transfected with LTR-Env, LTR-Env plus Ctr. RNA, or LTR-Env plus Ubc9 RNAi or left untransfected (U). The cells were pulsed (P) with [^{35}S]methionine for 45 min and then chased for 2 and 4 h. HIV-1 proteins were immunoprecipitated from cell lysates with AIDS patient sera. (b) Immunoprecipitated gp160 and gp120 bands were quantitated using Discovery Series Quantity One software, and the percentages of gp160 processed were calculated at 2 and 4 h. The error bars indicate standard deviations. (c) Summary of proteins involved in Env stability.

trans-dominant negative. Endogenous SUMO-1 is the limiting factor for SUMOylation (14); thus, overexpression of exogenous EGFP–SUMO-1 proteins greatly increases the amount of SUMOylated proteins detected by immunoblotting. A fourfold overexpression of Myc-C93A over endogenous Ubc9 led to an approximately 70% decrease in the global amount of EGFP-SUMOylated proteins. Quantitation of the 80-kDa band demonstrated a 90% decrease compared to the control (Fig. 7a). This result suggests that overexpression of Myc-C93A can inhibit SUMOylation effectively.

To examine if the SUMOylation activity of Ubc9 is important for HIV-1 infectivity, 293T cells were cotransfected with pNL4-3 and either pCMV-Tag3A (Fig. 7b, set 1), Myc-Ubc9 (Fig. 7b, set 2), or Myc-C93A (Fig. 7b, set 3). As expected, immunoblot analysis of whole-cell lysates and virions pelleted from the media of transfected cells showed that the overexpression of either Ubc9 or C93A had no effect on virion budding, maturation, or release (Fig. 7b). We then further examined whether the virions produced in the presence of the C93A mutant displayed decreased infectivity. As in previous experiments, the released virions were normalized for p24 content, and equal numbers of virions were used to infect TZM-bl indicator cells. In contrast to the loss of virion infectivity observed in the absence of Ubc9, overexpression of C93A and inhibition of SUMOylation in the producer cells had little effect on virion infectivity compared to control samples (Fig. 7c). In addition, Gag trafficked normally to perinuclear sites and colocalized with C93A Ubc9 (Fig. 7d). Collectively, these results suggest that Ubc9 may function independently of its E2 SUMO-conjugating activity during the late stages of the HIV-1 life cycle.

DISCUSSION

In this study, we have shown that Ubc9 interacts with the full-length HIV-1 Gag protein and have demonstrated for the first time that Ubc9 plays a role during the late stages of virus replication and that its expression in HIV-1-producing cells is important for conferring full infectivity on the nascent virus stock. Our results indicate that Ubc9 influences the stability and incorporation of mature Env into budding particles and that it may function during the HIV-1 assembly process in a SUMO-independent manner. These conclusions are supported by the following: (i) Ubc9-GST fusion proteins can interact with in vitro-translated Gag through its NC-p6 domain; (ii) Gag-Ubc9 complexes can be coimmunoprecipitated from cell lysates; (iii) Gag, Ubc9, and *trans*-dominant-negative C93A colocalize to discrete locations in the cytoplasm; (iv) overexpression of Ubc9 or C93A does not affect virion infection; (v) Ubc9 silencing leads to impaired virion infectivity; (vi) Ubc9 silencing has no effect on Gag assembly, budding, and processing or on Gag-mediated packing of the vRNAs, Vif, Vpr, Vpu, or the cellular protein cyclophilin A; (vii) Ubc9 is important for proper Env stability and incorporation of mature Env glycoproteins into virion particles when Env is expressed in the context of wt HIV-1 replication; and (viii) Ubc9 and Env do not appear to colocalize at the distinct perinuclear foci that contain Ubc9 and Gag.

While most studies that have characterized Env processing and stability were carried out in Env-only expression systems, our data suggested that in the context of wt HIV-1, Gag and Ubc9 are important for Env stability and incorporation into nascent virions. It was unexpected that the knockdown of a Gag-interacting partner would cause a change in Env stability and packaging, but it was even more so to find that this phenotype was directly tied to the presence or absence of Gag expression. While many have speculated that Env trimers interact with Gag at budding sites, biochemical and genetic evidence suggests that the initial Gag-Env interactions, whether direct or indirect, occur prior to their arrival at the assembly sites (13, 41–43, 52).

The mechanism by which Gag influences gp120 stability in the absence of Ubc9 has not been elucidated, but our data suggest that the Gag/Ubc9 complex could interact/affect Env after it has passed through the *trans*-Golgi, possibly in secretory vesicles, the plasma membrane, or recycling endosomes. Our data suggest that Ubc9 is not involved directly with Env processing, as gp160 processing and gp120 stability are normal in the absence of Gag and Ubc9, and Env and Ubc9 do not strongly colocalize in the same subcellular compartments. Although the majority of Ubc9-Gag colocalization is at perinuclear foci, which may be the Ubc9/SUMO-1-positive cellular

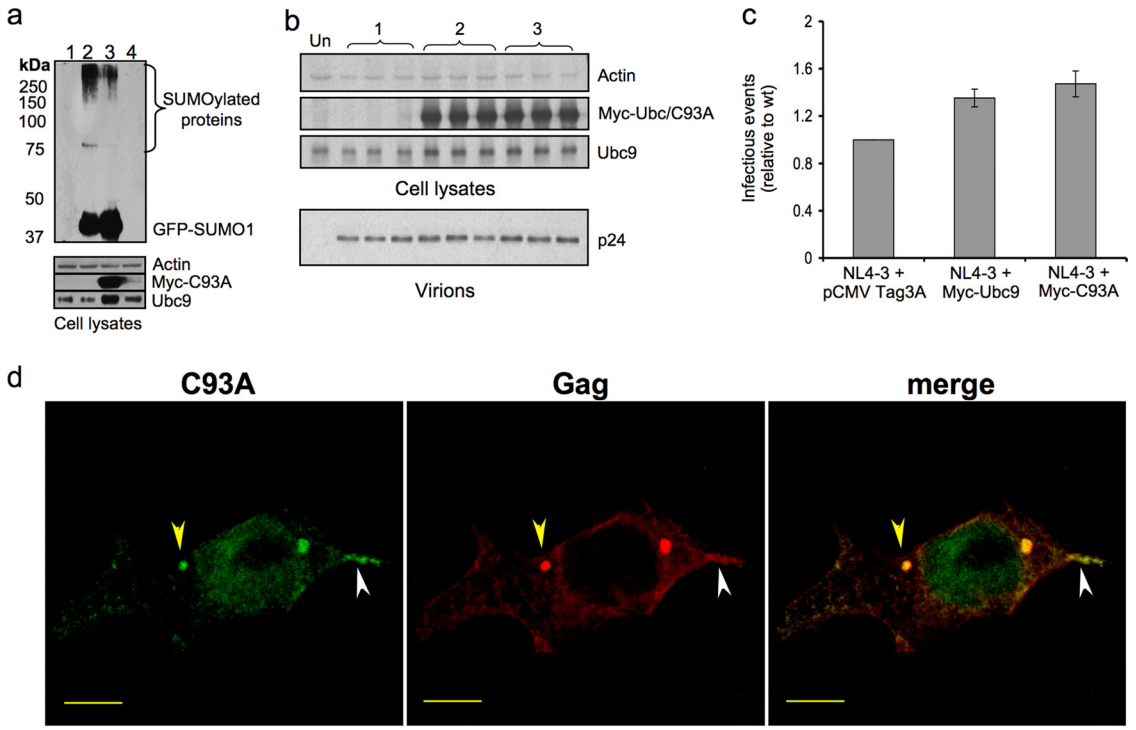

FIG. 7. Ubc9 may function independently of its E2 SUMO-conjugating activity during HIV-1 assembly. (a) Myc-C93A is a *trans*-dominant-negative active-site mutant and suppressed SUMOylation of cellular proteins. 293T cells were cotransfected with pEGFP-SUMO-1 and either pCMV-Tag3A (lane 2) or Myc-C93A (lane 3), with pCMV-Tag3A alone (lane 4), or left untransfected (lane 1). The cells were washed with PBS, lysed in SUMO lysis buffer, and boiled for 10 min. The lysates were assayed by immunoblotting using antibodies against SUMO-1 and Ubc9. (b) Overexpression of Myc-Ubc9 or Myc-C93A does not affect virion budding. 293T cells were cotransfected with pNL4-3 and either PCMV-Tag3A (sample set 1), Myc-Ubc9 (sample set 2), or Myc-C93A (sample set 3) or left untreated (Un). The media were harvested, and virions were pelleted through a 20% sucrose cushion. Virion budding and release were assayed by immunoblotting using antibodies against p24. The transfected cells were lysed directly in PSB, and protein expression was analyzed by immunoblotting using antibodies against Ubc9 and actin. (c) Overexpression of Myc-Ubc9 or Myc-C93A does not affect HIV-1 infectivity. Medium from transfected cells was harvested, and equal amounts of virus were used to infect TZM-bl indicator cells, as in previous experiments. The error bars indicate standard deviations. (d) HIV-1 Gag and Myc-C93A do not affect Gag trafficking or colocalization with Ubc9 at perinuclear sites. Transfected 293T cells were fixed and processed for immunofluorescence analysis by confocal microscopy, using anti-Ubc9 PAb and anti-p24 MAb. Cy5-conjugated donkey anti-mouse and Cy2-conjugated donkey anti-rabbit antibodies were used as secondary antibodies. Colocalization was analyzed using 0.3-μm optical sections. Representative medial sections are shown. The yellow and white arrowheads indicate areas of Ubc9 and Gag colocalization at perinuclear puncta and near the plasma membrane, respectively. Scale bars, 10 μm.

structures through which GPCs transiently traffic during an early stage of assembly (44), colocalization was observed in areas adjacent to the plasma membrane with both Ubc9 and C93A, as well. It is possible that this area is a functional subcellular site of interaction between Ubc9 and Gag. The close proximity of this colocalization site to the plasma membrane and the physical distance from the distinct perinuclear foci containing Gag and Ubc9 suggest that Ubc9 may also function at a later stage of assembly, a stage in which Gag has already trafficked through assembly intermediates and has been targeted to and is in close proximity to the plasma membrane, possibly at virus assembly sites.

The trafficking of Gag to the sites of Ubc9 colocalization is most likely very transient, as overexpression of Myc-Ubc9 greatly increases the amount of Gag localization to perinuclear sites containing Ubc9 but does not lead to a change in the amount of Gag released into the medium as virions. If Gag trafficking to sites of Ubc9 colocalization were not transient or Gag trafficking to assembly sites slowed, we would have expected a difference between the numbers of virions released from cells when Ubc9 or C93A was overexpressed. However, this phenotype was not observed, suggesting very transient Gag trafficking through the sites of Ubc9 colocalization. The full extent of how Ubc9 functions during the HIV-1 life cycle is still not well understood, and it may be involved with various steps during assembly.

Ubc9 is an E2 SUMO conjugase that is part of the cytoplasmic portion of the nuclear pore complex (77). It appears to localize to distinct, yet undefined, perinuclear regions and the secretory pathway and is also free in the cytoplasm (44, 62, 71, 74). Ubc9, through its SUMOylation activity, is involved in the targeting of transcription factors, cellular receptors, and viral proteins (reviewed in references 19, 26, and 45). However, there are a number of reports in the literature demonstrating that Ubc9 functions in a SUMO-independent manner. Androgen receptor (8), MEKK1, type I tumor necrosis factor alpha receptor (59), GLUT4 (40), RNA helicase A (3), glucocorticoid receptors (32), Vsx-1 (36), SOX4 (53), and human herpesvirus 6 IE2 (69) require Ubc9 binding for their functions and correct intracellular targeting, but they are not targets for SUMO-1 modification.

Our experiments with the dominant-negative C93A support a model in which Ubc9 functions as an essential binding part-

ner of Gag in the late HIV-1 life cycle but may do so independently of its SUMOylation conjugation function. If SUMOylation was important during the late stages of the HIV-1 life cycle, we would have expected to see a difference in virion infectivity with the *trans*-dominant C93A mutant compared to virions produced in control cells, yet little difference was observed. This model is supported by data reported by Gurer et al., in which the mutant HIV-1_{K27R} displayed no defects in virion assembly, even though it contained a mutated version of an in vivo SUMOylation site within p6. We have attempted to detect SUMOylated Gag or other viral proteins in both cell lysates and released virions at steady-state levels and when Ubc9 and SUMO-1 have been overexpressed but have been unsuccessful. It is interesting that p6 (K27R) showed diminished Ubc9 binding in a yeast two-hybrid system (21), but the mutation did not abolish its interaction with Ubc9 completely. Because of this, we carried out GST-Ubc9 pull-down experiments with K27R in the context of Pr55 and found that p6 (K27R) can still interact with Ubc9 in vitro (data not shown). This result suggests that there is more than one Ubc9 binding site within Gag or that mutation of lysine 27 in p6 is not enough to completely abolish Ubc9 binding.

In this study, we mainly focused on the effects of Ubc9 on the late events of the viral life cycle in producer cells. Even though Ubc9 is not packaged into virions at steady-state levels or when Ubc9 is overexpressed (data not shown), it has been reported that SUMO-1 plays a role during the early infectious events of HIV-1 (21, 76) and that it may regulate various steps within the retrovirus life cycle. To further understand the mechanisms by which Ubc9 functions in the HIV-1 life cycle, studies following Gag and Env trafficking in the absence of Ubc9 are currently under way. We are also investigating whether Ubc9 plays a direct role during the early infectious events of HIV-1 in target cells after entry. Our data show that Ubc9 plays an important role during HIV-1 replication, and we have identified another protein/pathway that could potentially be developed into novel antiretrovirals.

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