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Ubiquitin Is Covalently Attached to the p6^{Gag} Proteins of Human Immunodeficiency Virus Type 1 and Simian Immunodeficiency Virus and to the p12^{Gag} Protein of Moloney Murine Leukemia Virus

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Host proteins are incorporated into retroviral virions during assembly and budding. We have examined three retroviruses, human immunodeficiency virus type 1 (HIV-1), simian immunodeficiency virus (SIV), and Moloney murine leukemia virus (Mo-MuLV), for the presence of ubiquitin inside each of these virions. After a protease treatment to remove exterior viral as well as contaminating cellular proteins, the proteins remaining inside the virion were analyzed. The results presented here show that all three virions incorporate ubiquitin molecules at approximately 10% of the level of Gag found in virions. In addition to free ubiquitin, covalent ubiquitin-Gag complexes were detected, isolated, and characterized from all three viruses. Our immunoblot and protein sequencing results on treated virions showed that approximately 2% of either HIV-1 or SIV p6^{Gag} was covalently attached to a single ubiquitin molecule inside the respective virions and that approximately 2 to 5% of the p12^{Gag} in Mo-MuLV virions was monoubiquitinated. These results show that ubiquitination of Gag is conserved among these retroviruses and occurs in the p6^{Gag} portion of the Gag polyprotein, a region that is likely to be involved in assembly and budding.

Several studies of purified human immunodeficiency virus type 1 (HIV-1) virions have shown that in addition to proteins encoded by the virus, cellular proteins from the host are found in these particles (reviewed in reference 35). While some of these proteins may be taken into the virion simply because of their proximity to the viral assembly and budding sites, other cellular proteins are likely to be included in particles as a result of their interaction with viral proteins during assembly and release. Additionally, some host proteins may be incorporated to provide a function for the virus during the infection process. Given the possible interactions between viral and cellular proteins, the identification and study of the host proteins inside the HIV-1 virion are important for a better understanding of the HIV-1 virion structure, the viral life cycle, and virus-host interactions.

Ubiquitin has been found in highly purified HIV-1$_{MN}$ produced from lymphoid cells (1). Since ubiquitin has been found to be present inside the avian leukosis virus (40), a type C retrovirus, the association of ubiquitin with purified HIV-1 preparations strongly suggests that this protein is inside these virions as well.

Ubiquitin is found in all eukaryotes and appears to function in several important cellular processes (reviewed in references 7, 8, 13, 24, 27). Inside the cell, the majority of ubiquitin is found either as free ubiquitin or as long polymeric chains that are covalently attached to internal lysines in a wide range of cellular proteins (7, 8, 13, 24, 27). This modification appears to be specific and is thought to mark the protein for degradation by the 26S proteosome (reviewed in reference 16), though it is likely to play other, less characterized roles as well (25). The ubiquitin pathway has been found to be involved with many diverse cellular functions: cell cycle control, antigen presentation, heat shock response, receptor signaling, transcriptional activation, and DNA repair (7, 8, 13, 24, 27).

Though ubiquitin can be readily found in highly purified HIV-1, these results do not demonstrate that ubiquitin is truly present inside the virion, since even preparations that have been extensively purified by density gradient centrifugation contain microvesicles that copurify with virions (3, 15). These particles contain cellular proteins that can constitute a significant proportion of the total protein in the virus preparation (3, 15). In addition to contamination by microvesicles, it is also possible that cellular proteins from debris present in the culture supernatant may adhere to the exterior of the viral lipid envelope. These two possibilities make it difficult to conclude whether any cellular protein, in this case ubiquitin, is indeed inside HIV-1 particles. To provide a clearer picture of the proteins inside the virion, we have used a protease digestion technique that removes greater than 95% of the proteins associated with microvesicles, as well as nearly all of the proteins from the surface of the virus, yet preserves the proteins on the interior of the virus (36, 37). This technique was used on preparations of HIV-1 as well as two other important prototypic viruses, simian immunodeficiency virus isolate Mne (SIV$_{Mne}$) and Moloney murine leukemia virus (Mo-MuLV). Analyses of these treated virions show that ubiquitin is present inside each of these different retroviruses. Furthermore, immunoblot, high-pressure liquid chromatography (HPLC), and protein sequence data also demonstrated that each virus has a

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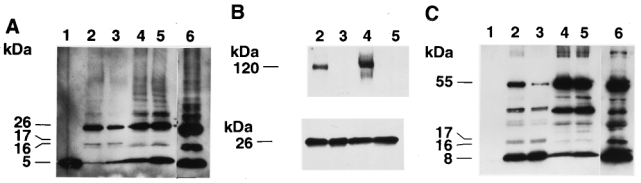

FIG. 1. Immunoblot analysis of digested HIV-1. (A) Immunoblot reacted with the antiubiquitin monoclonal antibody; (B) $gp120^{SU}$ (top) and $p24^{CA}$ (bottom) immunoblots of the material analyzed in panel A; (C) $p6^{Gag}$ immunoblot analysis of the blot presented in panel A after being stripped of the antiubiquitin reagents. Lane 1, 25 ng of purified ubiquitin; lanes 2 and 3, 20 μg of mock-digested and digested $HIV\text{-}1_{MN}$ produced from Clone 4-13, respectively; lanes 4 and 5, 20 μg of mock-digested and digested $HIV\text{-}1_{NL4\text{-}3}$ produced from H9 cells, respectively. Molecular masses of bands are indicated at the left.

small amount of a mature Gag cleavage product that is conjugated (i.e., covalently linked) to ubiquitin.

MATERIALS AND METHODS

Virus production. Virus was produced from infected cell lines as follows: $HIV\text{-}1_{MN}$ was produced from the lymphoid cell lines H9, Cem_{SS}, and Clone 4-13 (a subclone of the previously described Clone 4 cell line, a chronically infected HIV-1-infected H9 cell line that has been single-cell cloned [38]); $HIV\text{-}1_{NL4\text{-}3}$ was produced from acutely infected H9 cells; SIV_{Mne} was produced from the HuT78 T-lymphocyte line; and Mo-MuLV was produced from the NIH 3T3 mouse embryo fibroblast line. $HIV\text{-}1_{NL4\text{-}3}$ was ultracentrifuged from the culture supernatant by a single centrifugation step though 20% sucrose in an SW28Ti rotor (Beckman Instruments, Palo, Alto, Calif.) at 25,000 rpm for 1 h at 4°C. All of the other virions were purified from tissue culture supernatants of infected cell cultures by banding in two successive sucrose density gradients as previously described (4).

Protein analysis. Digested virions were produced by treating virus at concentrations of 1 to 3 mg of protein (as measured by the method of Lowry et al. [30]) with 1 mg of subtilisin (Boehringer Mannheim, Indianapolis, Ind.) per ml for 18 h at 37°C. As a control, the same amount of virus sample used for the digested sample was mock digested (i.e., processed the same way as the digested samples except without the addition of protease during the 37°C incubation). The treated virus preparations were then separated from the digestion mixture and protein fragments by sucrose density centrifugation as previously described (36, 37). Reverse-phase HPLC and N-terminal protein sequence analysis of the virions were performed as previously described (21). Immunoblot analysis was performed as previously described, using the enhanced chemiluminesce procedure (Amersham Life Science, Arlington Heights, Ill.) (22). Rabbit antiserum against ubiquitin was obtained from Sigma (St. Louis, Mo.); monoclonal antibody against ubiquitin was obtained from Chemicon (Temecula, Calif.); rabbit antiserum against ubiquitin cross-reacting protein was a kind gift from Arthur Haas, Medical College of Wisconsin, Milwaukee); goat antiserum against HIV-1 $p6^{Gag}$ was obtained from the AIDS Vaccine Program; SIV $p6^{Gag}$ antiserum was a kind gift from Raoul Benveniste, Laboratory of Viral Carcinogenesis, NCI-FCRDC.

Estimation of relative amounts of protein. The relative amount of either ubiquitin or ubiquitin-Gag complex versus Gag was estimated by comparing the peak areas from the HPLC chromatogram A_{206} profiles. The areas, after adjustment for molecular weight differences, were compared as follows: the free ubiquitin-containing peaks were compared to the $p17^{MA}$ (matrix protein) peak for HIV-1, the $p18^{MA}$ peak for SIV, and the $p30^{CA}$ (capsid protein) peak for Mo-MuLV. The areas of the peaks containing ubiquitin-Gag conjugates were compared to those of the respective unmodified mature Gag proteins. Estimations were also based on the individual molar amino acid yields for ubiquitin and ubiquitin complexes as detected by the protein sequence analysis. Finally, these relative results were compared with the signals from our immunoblot data for agreement with our estimations.

RESULTS

To determine if ubiquitin is inside HIV-1 virions, viral preparations of the $HIV\text{-}1_{MN}$ and $HIV\text{-}1_{NL4\text{-}3}$ strains were digested with the nonspecific protease subtilisin, and the treated virions were repurified. Ubiquitin immunoblot analysis of both digested and mock-digested virions detected nearly equal levels of a 5-kDa band in the two samples (Fig. 1A). While the apparent size of ubiquitin by the immunoblot analysis was less than the expected 8.5 kDa, commercially obtained ubiquitin also migrated at this anomalous size (Fig. 1A), indicating that the ubiquitin molecules incorporated into the virus were either full length or nearly full length. Experiments with purified ubiquitin have determined that this protein was readily digested by subtilisin under the conditions used for the virus treatment (data not shown). Additional immunoblot analysis of digested viral samples with antiserum against $p24^{CA}$ showed that the levels of this interior viral protein were unchanged, while blotting with antiserum against $gp120^{SU}$ confirmed that exterior proteins were removed from the treated preparations (Fig. 1B). Since ubiquitin is protected from digestion, apparently by the lipid viral envelope, these data suggest that the majority of the ubiquitin found in these HIV-1 preparations is present inside the virion.

In addition to the 5-kDa band in the ubiquitin immunoblot, two reacting bands are present at 16 and 17 kDa in all of the HIV-1 samples. Based on size, these bands could be ubiquitin conjugated to one of the small Gag proteins, either $p7^{NC}$ (nucleocapsid) or $p6^{Gag}$. To test this hypothesis, the ubiquitin immunoblot was stripped of the antiubiquitin reagents and reprobed with antiserum against $p6^{Gag}$. This experiment, shown in Fig. 1C, detected the $p6^{Gag}$ cleavage protein, migrating at 8 kDa, as well as several higher-molecular-mass forms, most of which are likely to be the various incomplete Gag polyprotein cleavage products (e.g., the 55-kDa Gag polyprotein). However, in addition to these expected higher-molecular-mass bands, the 16- and 17-kDa bands that were seen by the antiubiquitin staining were also detected by the $p6^{Gag}$ antiserum (Fig. 1). Parallel experiments to look for complexes between ubiquitin and $p7^{NC}$ by immunoblot analysis did not detect these same or other higher-molecular-mass forms that comigrated with bands in the ubiquitin immunoblot but did detect a smaller (approximately 15-kDa) form that could be the $p7^{NC}$-p1-$p6^{Gag}$ proteolytic processing intermediate (data not shown). Therefore, it appears that in addition to free ubiquitin, HIV-1 contains a covalent $p6^{Gag}$-ubiquitin complex inside the viral particle. The presence of two bands at 16 and 17 kDa in these immunoblots band is probably due to alternative processing of $p6^{Gag}$ by the HIV-1 protease. Previous studies have shown that a fraction of the $p6^{Gag}$ protein within $HIV\text{-}1_{MN}$ virions is cleaved internally by the HIV-1 protease, resulting in the removal of 16 amino acids from C terminus of $p6^{Gag}$ (21, 34). This proteolytic processing reduces the molecular size of $p6^{Gag}$ by 2 kDa. Therefore, the 16-kDa coreacting band in these immunoblots is likely to be a complex between this truncated $p6^{Gag}$ species and a single ubiquitin.

In addition to bands at 5, 16, and 17 kDa, the ubiquitin immunoblot shows a prominent band at 26 kDa (Fig. 1A). This band also coreacted with $p24^{CA}$ antiserum (Fig. 1B and data not shown), indicating that this ubiquitin antibody may nonspecifically react with the $p24^{CA}$ from the virus. It is important to consider that these blots contain large amounts of virus that may be a cause of this CA artifact. (Our experience with immunoblots has been that many specific monoclonal antibodies will nonspecifically stick to the relatively hydrophobic retroviral capsid proteins when large amounts of viral protein are loaded [9].) Experiments with HPLC-purified $p24^{CA}$ have shown that the monoclonal antibody against ubiquitin also detects $p24^{CA}$ when present in relatively large quantities, while immunoblots of much smaller amounts of virions do not exhibit this strong CA-reacting artifact (data not shown).

Some other faint higher-molecular-weight bands are also visible in the ubiquitin blot (Fig. 1A). None of these bands clearly comigrated with bands detected by immunoblotting for other Gag proteins (data not shown), suggesting that ubiquitin is not conjugated to significant amounts of the other mature Gag proteins. Therefore, even though these large proteins that

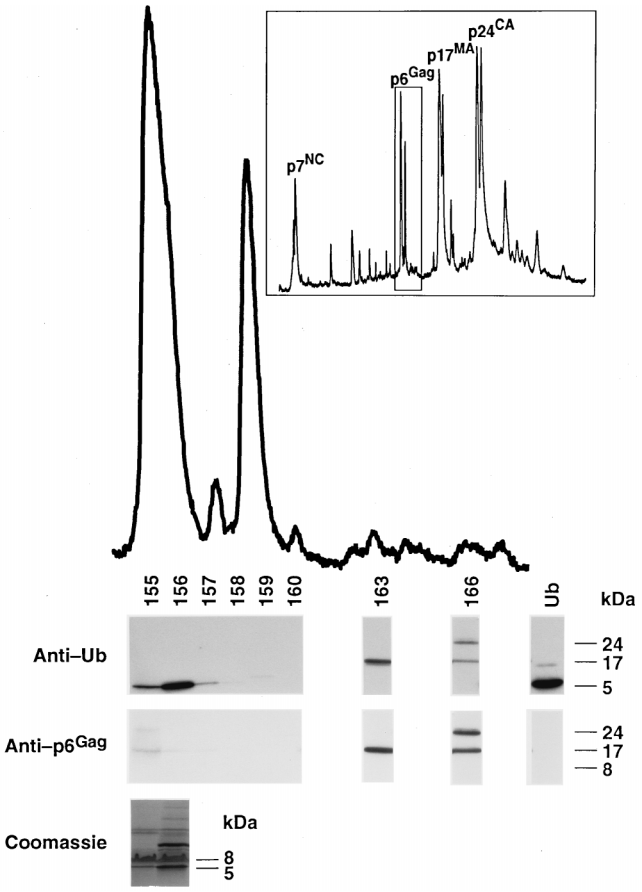

FIG. 2. HPLC analysis of ubiquitin in a digested HIV-1 preparation. The region of the HIV-1 HPLC analysis that contains ubiquitin is presented with the results of immunoblot and C-SDS-PAGE analysis of selected fractions arranged under the corresponding regions of the chromatogram. The complete chromatogram is placed in an inset with a box around the region presented here. The antiserum or stain used for each set of results is labeled on the left of the corresponding blot or gel; sizes of the bands as determined by relative mobility are presented on the right. The antiserum to ubiquitin (Anti-Ub) was used for these ubiquitin immunoblots.

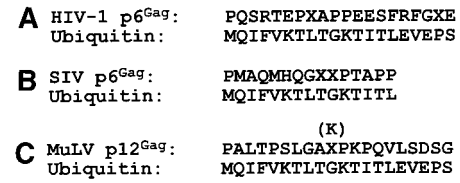

FIG. 3. Protein sequence results for ubiquitin-Gag conjugates. Sequences are presented in the standard single-letter amino acid code, and X indicates an ambiguous or missing residue. (A) Protein sequence results from the 17-kDa putative ubiquitin-p6^{Gag} band that was HPLC purified from HIV-1; (B) protein sequence results from the 16-kDa putative ubiquitin-p6^{Gag} band that was HPLC purified from SIV$_{Mne}$; (C) protein sequence results from the 20-kDa putative ubiquitin-p12^{Gag} band that was HPLC purified from Mo-MuLV. The missing lysine residue in the 20-kDa sequence is indicated by X within the p12^{Gag} sequence, with the missing lysine indicated above.

are detected in the ubiquitin immunoblot have not been identified, they are likely to be ubiquitin complexed to cellular rather than viral proteins.

HPLC analysis of ubiquitin and p6^{Gag}-ubiquitin conjugates. To analyze further the free ubiquitin and the ubiquitin-Gag complexes present inside HIV-1, 100 mg of HIV-1$_{MN}$ virus produced from Clone 4-13 cells was treated with subtilisin, and the digested particles were repurified and then subjected to HPLC analysis. The resulting fractions were examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by Coomassie blue staining (C-SDS-PAGE), immunoblotting, and protein sequence analysis for the presence of ubiquitin. The results from ubiquitin immunoblotting of selected fractions (Fig. 2) demonstrated that ubiquitin was present as a 5-kDa band in fractions 155 and 156, with the majority of the protein present in fraction 156. This result was confirmed by C-SDS-PAGE (Fig. 2) and protein sequencing of the 5-kDa band that produced a ubiquitin sequence. This gel also revealed that a protein with an apparent size of 8 kDa accounted for the majority of the protein in fractions 155 and 156 (Fig. 2). Immunoblotting and sequence analysis determined that this protein is the p6^{Gag} cleavage product (data not shown). (The blot in Fig. 2 did not show appreciable signal for p6^{Gag} because the conditions used for this particular blot did not adequately bind the relatively hydrophilic p6^{Gag} protein to the blotting membrane.) These two observations show that ubiquitin elutes in the trailing half of the p6^{Gag} peak in this HPLC analysis. The HPLC A_{206} profile allows us to estimate that compared to the amount of p17^{MA} present in the digested preparation, free ubiquitin molecules are present inside HIV-1 virions at approximately 10% of the Gag protein level. This estimate is consistent with the amounts of ubiquitin detected by protein sequencing, C-SDS-PAGE, and immunoblot analysis.

In addition to free ubiquitin, fractions from this region of the HPLC were found to contain the covalent ubiquitin-Gag complex identified by the immunoblot analyses presented in Fig. 1. Ubiquitin immunoblot analysis of fractions 163 and 166 revealed bands with an apparent molecular size of 17 kDa that, after stripping and reprobing, also were detected by the anti-p6^{Gag} antiserum. (Unlike p6^{Gag} alone from fractions 155 and 156, these covalent Gag-ubiquitin complexes readily adhere to the blotting membrane by virtue of their ubiquitin sequences.) While the two forms of this protein that were seen in whole-virus preparations are not readily apparent in these blots, it is possible that the amount of the ubiquitin-p6^{Gag} protein in this blot (being much higher than the whole-virus samples) causes both the 16- and 17-kDa bands to appear to migrate as one on the immunoblot. Immunoblot analysis of this region with p7^{NC} antiserum did not detect any bands that comigrate with those detected in the ubiquitin or p6^{Gag} immunoblots.

Protein sequence analysis of the 17-kDa protein that was purified by HPLC from an undigested HIV-1$_{MN}$ preparation yielded two equimolar N-terminal sequences for both p6^{Gag} and ubiquitin sequences (Fig. 3A). Since these sequences are produced simultaneously from a single 17-kDa band that was isolated by reducing SDS-PAGE, this result shows that the protein has two amino termini, one ubiquitin and the other p6^{Gag}, directly demonstrating the presence of a covalent ubiquitin-p6^{Gag} complex. Sequencing of other similar-sized bands from the HPLC fractions did not detect any p7^{NC}-ubiquitin conjugate. Therefore, these data and those from the immunoblots presented in Fig. 1 show that this 17-kDa protein (and likely the 16-kDa protein detected in the whole-virus immunoblot [Fig. 1]) is a complex of p6^{Gag} that is covalently attached to a single ubiquitin. Based on these data, we estimate that approximately 2% of p6^{Gag} is modified by ubiquitin.

In addition to a 17-kDa band, both ubiquitin and p6^{Gag} immunoblot analysis of the HPLC fractions 165 and 166 detected an additional 24-kDa coreacting band. Unfortunately, there is not enough of this coreacting protein for sequence

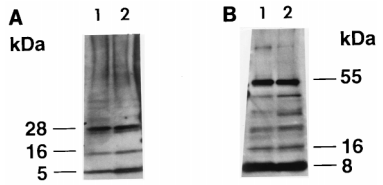

FIG. 4. Immunoblot that was reacted with the antiubiquitin monoclonal antibody (A) then stripped and reprobed with anti-SIV $p6^{Gag}$ (B). The samples were 40 μg of total protein of mock-digested (lane 1) and digested (lane 2) preparations of SIV_{Mne}. Molecular masses of bands are indicated on the left.

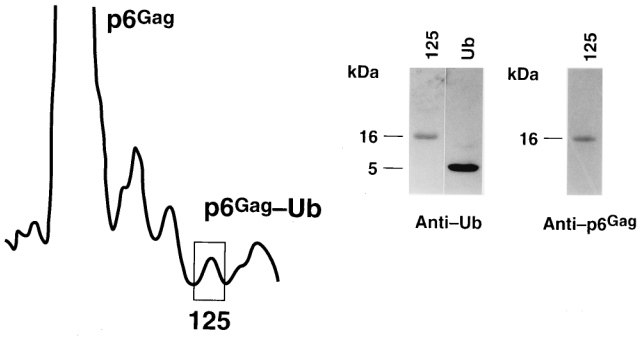

FIG. 5. HPLC purification of a putative ubiquitin-SIV $p6^{Gag}$ conjugate from SIV. The HPLC profile of the ubiquitin (Ub)-containing region is presented along with antiubiquitin (using the antiubiquitin antiserum) and anti-SIV $p6^{Gag}$ immunoblot analysis of fraction 125. Molecular masses of bands are indicated on the left.

analysis. The size of this conjugate is consistent with that of a $p6^{Gag}$ molecule which is modified with two ubiquitin molecules (6 kDa + 2 × 8.5 kDa), though there are at least two other possible structures that would produce similar-size bands in these immunoblots. A complex of $p6^{Gag}$ conjugated to the 15-kDa ubiquitin cross-reacting protein (UCRP) would also produce a protein with an apparent size similar to the 24 kDa that is observed. Like ubiquitin, UCRP is a protein that is conjugated to an internal lysine in cellular proteins and commonly cross-reacts with antibodies made to ubiquitin due to their sequence similarities (29). However, immunoblot experiments using a specific antiserum against UCRP failed to detect the 24-kDa band, showing that UCRP does not appear to be attached to $p6^{Gag}$ (data not shown). Another alternative ubiquitin-Gag protein complex that could generate a product close to the observed size of 24 kDa is a complex between ubiquitin (8.5 kDa) and an uncleaved $p7^{NC}$-p1-$p6^{Gag}$ cleavage product (15 kDa). However, $p7^{NC}$ immunoblot analysis of fraction 166 failed to detect this band, indicating that this putative structure is not present (data not shown). Taken together, these data indicate that the 24-kDa protein is most likely one $p6^{Gag}$ molecule covalently linked to two ubiquitin molecules. Due to the trace amounts present, our data do not allow for a good estimate for the amount of this complex present inside virions; however, it is likely to be very low, much less than 1% of the total $p6^{Gag}$.

Ubiquitin is present in SIV_{Mne} virions. The analysis of ubiquitin inside retroviral particles was extended to SIV. Antiubiquitin immunoblot analysis of equal amounts of digested and mock-digested samples from an SIV_{Mne} virion preparation showed that SIV, like HIV-1, contains ubiquitin (Fig. 4). A comparison of the digested sample with the mock-digested control indicates that most of the ubiquitin was protected from digestion and thus is inside the virion. Based on the immunoblot analysis and comparison of the ubiquitin and $p6^{Gag}$ HPLC profiles of SIV (data not shown), free ubiquitin molecules appear to be present at approximately 10% of SIV Gag protein level. In addition to the 5-kDa ubiquitin band, higher-molecular-mass bands of 16 and 28 kDa are also seen in the immunoblot. Given the relative similarity between HIV-1 and SIV and the likelihood that their respective $p6^{Gag}$ molecules provide the same function for both viruses, this 16-kDa band is probably a ubiquitin-$p6^{Gag}$ complex. To test this possibility, the ubiquitin immunoblot was stripped and reprobed with antiserum against SIV $p6^{Gag}$. The results, shown in Fig. 4, revealed that the same 16-kDa band that reacted in the ubiquitin immunoblot also reacted with the SIV $p6^{Gag}$ antiserum, indicating that like HIV-1, SIV contains a complex between its $p6^{Gag}$ and a single ubiquitin.

The other prominent reacting band in the ubiquitin immunoblot at 28 kDa also reacted with $p28^{CA}$ antiserum (Fig. 4); therefore, this band appears to be $p28^{CA}$ (data not shown) that is detected nonspecifically due to the large amounts of protein present on the blot, similar to the result for HIV-1 $p24^{CA}$. Protein sequence analysis of this 28-kDa reacting band produced only $p28^{CA}$ sequence and no ubiquitin or UCRP sequence.

Fractions from an HPLC analysis of a 200-mg SIV_{Mne} preparation were examined for the presence of the SIV $p6^{Gag}$-ubiquitin complex by C-SDS-PAGE, immunoblotting, and protein sequence analysis. Results from these methods detected a 16-kDa protein in HPLC fraction 125 that reacts in both ubiquitin and SIV $p6^{Gag}$ immunoblots (Fig. 5). N-terminal protein sequence analysis of the 16-kDa band produced equimolar sequences of both $p6^{Gag}$ and ubiquitin proteins (Fig. 3B), directly demonstrating the presence of this Gag-ubiquitin complex. Unlike the results for HIV-1, a diubiquitinated form of SIV $p6^{Gag}$ was not detected in the SIV HPLC fractions. Taken together, all of these data show that SIV contains a single ubiquitin conjugated to $p6^{Gag}$. Our HPLC and protein sequence data indicate that approximately 2% of the $p6^{Gag}$ in SIV is modified by ubiquitin.

Ubiquitin in Mo-MuLV. Mo-MuLV, a prototypic simple retrovirus, was examined for free ubiquitin and evidence of ubiquitin conjugation to Gag. Ubiquitin immunoblot analysis of both mock- and protease-treated Mo-MuLV virions showed that the amounts of ubiquitin were equal in the two samples, demonstrating that ubiquitin is protected from protease digestion and thus likely to be inside the Mo-MuLV virions (Fig. 6). From the immunoblot, protein sequence, and HPLC data (data not shown), we estimate Mo-MuLV to contain free ubiq-

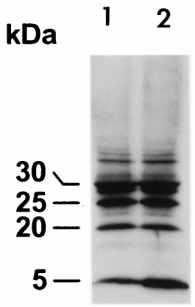

FIG. 6. Ubiquitin immunoblot of digested and mock-digested Mo-MuLV virions. The Mo-MuLV samples were 20 μg of total protein of mock-digested (lane 1) and digested (lane 2) preparations. The immunoblot was reacted with the antiubiquitin monoclonal antibody. Molecular masses are presented on the left.

uitin molecules at approximately 8% of the Gag protein level compared to the levels $p30^{CA}$ present in the virion preparation.

Other, higher-molecular-mass bands, especially a band at 30 kDa, were also present in the ubiquitin immunoblot. (The band at 30 kDa also reacted with $p30^{CA}$ antiserum [data not shown] and thus is likely to be another instance of a monoclonal antibody nonspecifically reacting with the hydrophobic capsid protein.) Another band present just below the $p30^{CA}$ appears by immunoblot and protein sequence analysis to be a truncated form of $p30^{CA}$ starting at proline 34 of the Mo-MuLV sequence. A short N-terminal sequence of the band at 25 kDa did not match the sequences within Mo-MuLV Gag or the N-terminus of ubiquitin. Its identity is unknown.

The 20-kDa band that was detected by immunoblot analysis with ubiquitin antiserum (Fig. 6) was also detected and isolated from HPLC-fractionated virions (data not shown). Protein sequence analysis of this single band produced equimolar sequences of both $p12^{Gag}$ and ubiquitin, establishing that this MuLV Gag cleavage product is modified with ubiquitin (Fig. 3C). The experimental protein sequence essentially matched the previously published sequenced for $p12^{Gag}$ except for a missing lysine residue at position 10 (Fig. 3C). This missing residue is not due to a technical peculiarity of the analysis since sequencing of unmodified $p12^{Gag}$ produced from the same HPLC separation readily detected the lysine at position 10 (data not shown). To interpret these data, it is important to understand that automated N-terminal protein sequencing cannot detect isopeptide-bonded amino acids. In the case of ubiquitinated proteins, the ε-amino group of the conjugated lysine is linked to the C-terminal carboxyl group of ubiquitin. When the primary peptide bond of that lysine is cleaved by the N-terminal degradation process, this isopeptide-bonded residue is not released from the solid support since it is still attached to the immobilized ubiquitin molecule. Therefore, a lysine that is covalently attached to ubiquitin is not released to the detector and is missing from the sequence. Thus, the absence of lysine 10 indicates that this $p12^{Gag}$ residue is linked to ubiquitin. The only other lysine in $p12^{Gag}$ is found at position 12, and there was no appreciable reduction in its signal during the sequence analysis. While the majority of the ubiquitination appears to be on lysine 10, the presence of a small fraction (~10%) of this complex that is modified at lysine 12 cannot be ruled out. Taken together, these data demonstrate that this 20-kDa band consists of a single ubiquitin conjugated to $p12^{Gag}$ mostly on lysine 10. Based on our HPLC and sequence data, we estimate that the approximately 2 to 5% of the $p12^{Gag}$ in Mo-MuLV virions is conjugated to ubiquitin.

DISCUSSION

We have shown by analysis of protease-digested virions that ubiquitin is present inside several different retroviruses, HIV-1, SIV, and Mo-MuLV. Analysis of digested HIV-1 showed that free ubiquitin protein was present in the virions at approximately 10% of the amount of Gag proteins. From immunoblot and HPLC analysis, both SIV and MuLV also appear to incorporate levels of ubiquitin that are similar to those found inside HIV-1. Ubiquitin incorporation does not appear to be cell type specific since it has been found in HIV-1 virions produced both from lymphoid cells and from transfected 293T human transformed kidney cells (10) as well as Mo-MuLV produced from mouse fibroblasts.

We also have found that each virus contained, in addition to free ubiquitin, a small fraction of a mature Gag protein complexed to a single ubiquitin. This is the first demonstration that ubiquitin is covalently attached to a retroviral Gag protein. For both HIV-1 and SIV, approximately 2% the $p6^{Gag}$ was conjugated to a single ubiquitin; approximately 2 to 5% of the $p12^{Gag}$ in MuLV was found to be monoubiquitinated. Since $p12^{Gag}$ contains only two lysines and both are present near the N terminus of the protein, sequence analysis of the $p12^{Gag}$-ubiquitin complex was able to determine that lysine 10 is the primary site of modification in the protein conjugate analyzed. Unfortunately, the modified sites for both HIV-1 and SIV $p6^{Gag}$ are not known since neither of these proteins contains any lysines near the N terminus, and there are insufficient quantities of the complexes for further internal sequence analysis.

In addition to a 17-kDa conjugate, a 24-kDa protein that reacted in both antiubiquitin and anti-$p6^{Gag}$ immunoblots was observed. Based on our HPLC and immunoblot data, we suggest that this faster-migrating form is a $p6^{Gag}$ that is modified with two ubiquitin molecules since the two other likely alternatives, $p6^{Gag}$ conjugated to UCRP and an incompletely cleaved $p7^{NC}$-p1-$p6^{Gag}$ product conjugated to ubiquitin, could not be experimentally demonstrated. Our analysis cannot distinguish whether this protein consists of tandem ubiquitin molecules attached to one lysine or of two single ubiquitin molecules attached to two different lysines. However, the former explanation is unlikely because ubiquitin appears to be almost exclusively attached to the target protein either singly or as multimers but rarely as dimers (7, 8, 14, 24, 27). Therefore, it is likely that this 24-kDa form of $p6^{Gag}$ has two lysines that are each conjugated to a single ubiquitin.

These results show that the ubiquitination of a small portion of Gag is common to all three of the retroviruses examined. Free ubiquitin has been previously found by Putterman et al. (40) to be associated with avian leukosis virions at levels (10%) similar to those that we have found inside HIV-1. However, a search for ubiquitinated Gag proteins by this group using immunoprecipitation and immunoblotting analyses failed to detect any complexes. This may be due to the potentially small amounts of modified protein, if any, that might be present. Alternatively, since there are many cellular enzymes that can cleave the isopeptide bond between ubiquitin and the modified protein (7, 8, 14, 24, 27), it is possible that ubiquitin was removed from the Gag proteins by an isopeptidase that was incorporated into the virion. Theoretically, it is possible that some of the avian leukosis virus Gag originally was conjugated to ubiquitin but was removed inside the virion and is detected as free ubiquitin in the analysis. In this light, there also might have been more Gag-ubiquitin conjugates initially incorporated into HIV-1, SIV, and Mo-MuLV than were detected here, but some of these complexes might have been cleaved within the virion or even during protein analysis. Considering that there are significant levels of free ubiquitin (up to 10% of Gag), there may have been considerably more covalent Gag-ubiquitin complexes initially incorporated into the virions than was found in this study.

The $p6^{Gag}$ protein is found at the C termini of both the HIV-1 and SIV Gag precursors (34). This region in HIV-1 appears to be involved in assembly and budding (19, 26, 39). Due to its position and sequence, the $p6^{Gag}$ region of SIV is likely to function in the same manner. The $p12^{Gag}$ protein of Mo-MuLV located between the $p15^{MA}$ and $p30^{CA}$ proteins in the $Pr65^{Gag}$ precursor. The function of this protein is in the MuLV life cycle is not understood.

Ubiquitin is specifically attached to proteins by a process that involves at least two steps (7, 8, 14, 24, 27). The specificity of the modification is produced by the interaction of ubiquitinating enzymes, in most cases one of many E_2 enzymes, with the substrate protein by a specialized protein-protein interac-

tion between the enzyme and its substrate (5, 18). Given this observation, it is unlikely that the enzymatic attachment ubiquitin to $p6^{Gag}$ or $p12^{Gag}$ occurs nonspecifically. If this were so, it might be expected that the lysine-rich NC protein would be more likely to have been modified. However, we found no evidence that NC is conjugated to ubiquitin. Therefore, it is more likely that these Gag regions in the assembling virions contact or mimic a cellular protein that is normally ubiquitinated.

Ubiquitin conjugation serves many roles in the cell and has recently been under intense study (25). The most common function for this system involves the attachment of polyubiquitin chains to the target protein that signals for it to be degraded by the proteosome. However, polyubiquitination has been shown to be a signal for processes other than degradation: it has been shown to be a required step for endocytosis of a plasma membrane receptor and for activation of a IκBα protein kinase complex (6, 23). In contrast, little is known about possible functions for the monoubiquitination of a cellular protein. Histones 2A and 2B are the most prominent cellular proteins that have been shown to have only one ubiquitin attached to them (18, 43). A role for these complexes has not been clearly demonstrated, though there appears to be a link between modified histones and transcriptionally active chromatin (28). The attachment of ubiquitin to these two histones appears to be reversible, the ubiquitin being attached and removed during the cell cycle by a mechanism that does not appear to result in increased protein turnover of these proteins (32). In addition to histones, the capsid proteins of several plant viruses have also been found to be modified by single ubiquitin proteins (12, 20). In these cases, only one of the approximately 2,000 capsid proteins in each virion appears to be modified by a single ubiquitin. Several cytoskeletal proteins, including actin, as well as members of the microtubule network have been found to be conjugated to a single ubiquitin molecule (2, 11, 33), though the functions of these modified proteins are not known. Retroviruses, especially HIV-1 and MuLV, appear to interact with the host cytoskeleton during assembly and budding (31, 37, 41, 42). Additionally, an interaction between Rous sarcoma virus Gag and a cellular protein during assembly and budding has been proposed (14). This suggests that the monoubiquitination of HIV-1, SIV, and Mo-MuLV Gag proteins might be a result of an interaction between these viruses and a cytoskeleton-associated ubiquitinating activity during assembly and budding.

The functional significance of ubiquitin and Gag-ubiquitin complexes in retroviruses is not clear. As the cellular functions for monoubiquitination emerge, a role for this Gag modification may be come clearer.

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REFERENCES

1. **Arthur, L. O., J. W. Bess, Jr., R. C. Sowder II, R. E. Benveniste, L. D. Mann, J.-C. Chermann, and L. E. Henderson.** 1992. Cellular proteins bound to immunodeficiency viruses: implications for pathogenesis and vaccines. Science **258:**1935–1938.
2. **Ball, E., C. C. Karlik, C. J. Beall, D. L. Saville, J. C. Sparrow, B. Bullard, and E. A. Fyrberg.** 1987. Arthrin, a myofibrillar protein of insect flight muscle, is an actin-ubiquitin conjugate. Cell **51:**221–228.
3. **Bess, J. W., Jr., R. J. Gorelick, W. J. Bosche, L. E. Henderson, and L. O. Arthur.** 1997. Microvesicles are a source of contaminating cellular proteins found in purified HIV-1 preparations. Virology **230:**134–144.
4. **Bess, J. W., Jr., P. J. Powell, H. J. Issaq, L. J. Schumack, M. K. Grimes, L. E. Henderson, and L. O. Arthur.** 1992. Tightly bound zinc in human immunodeficiency virus type 1, human T-cell leukemia virus type 1, and other retroviruses. J. Virol. **66:**840–847.
5. **Chau, V., J. W. Tobias, A. Bachmair, D. Marriott, D. J. Ecker, D. K. Gonda, and A. Varshavsky.** 1989. A multiubiquitin chain is confined to specific lysine in a targeted short-lived protein. Nature **243:**1576–1583.
6. **Chen, Z. J., L. Parent, and T. Maniatis.** 1996. Site-specific phosphorylation of IκBα by a novel ubiquitination-dependent protein kinase activity. Cell **84:**853–862.
7. **Ciechanover, A.** 1994. The ubiquitin-proteasome proteolytic pathway. Cell **79:**13–21.
8. **Ciechanover, A., and A. L. Schwartz.** 1994. The ubiquitin-mediated proteolytic pathway: mechanisms of recognition of the proteolytic substrate and involvement in the degradation of native cellular proteins. FASEB J. **8:**182–191.
9. **Coren, L. V., and L. E. Henderson.** Unpublished data.
10. **Coren, L. V., and D. E. Ott.** Unpublished data.
11. **Corsi, D., L. Galluzzi, R. Crinelli, and M. Magnani.** 1995. Ubiquitin is conjugated to the cytoskeletal protein α-spectrin in mature erythrocytes. J. Biol. Chem. **270:**8928–8935.
12. **Dunigan, D. D., R. G. Dietzgen, J. E. Schoelz, and M. Zaitlin.** 1988. Tobacco mosaic virus particles contain ubiquitinated coat protein subunits. Virology **165:**310–312.
13. **Finley, D., and V. Chau.** 1991. Ubiquitination. Annu. Rev. Cell Biol. **7:**25–69.
14. **Garnier, L., J. W. Wills, M. F. Verderame, and M. Sudol.** 1996. WW domains and retrovirus budding. Nature **381:**744–745.
15. **Gluschankof, P., I. Mondor, H. R. Gelderblom, and Q. J. Sattentau.** 1997. Cell membrane vesicles are a major contaminant of gradient-enriched human immunodeficiency virus type-1 preparations. Virology **230:**125–133.
16. **Goldberg, A. L.** 1995. Functions of the proteasome: the lysis at the end of the tunnel. Science **268:**522–523.
17. **Goldknoff, I. L., and H. Busch.** 1977. Isopeptide linkage between nonhistone and histone 2A polypeptides of chromosomal conjugate-protein A24. Proc. Natl. Acad. Sci. USA **74:**864–868.
18. **Gosink, M. M., and R. D. Vierstra.** 1995. Redirecting the specificity of ubiquitination by modifying ubiquitin-conjugating enzymes. Proc. Natl. Acad. Sci. USA **92:**9117–9121.
19. **Gottlinger, H. G., T. Dorfman, J. G. Sodroski, and W. A. Haseltine.** 1991. Effect of mutations affecting the p6 Gag protein on human immunodeficiency virus particle release. Proc. Natl. Acad. Sci. USA **88:**3195–3199.
20. **Hazelwood, D. and M. Zaitlin.** 1990. Ubiquitinated conjugates are found in preparations of several plant viruses. Virology **177:**352–356.
21. **Henderson, L. E., M. A. Bowers, R. C. Sowder II, S. Serabyn, D. G. Johnson, J. W. Bess, Jr., L. O. Arthur, D. K. Bryant, and C. Fenselau.** 1992. Gag proteins of the highly replicative MN strain of human immunodeficiency virus type 1: posttranslational modifications, proteolytic processings, and complete amino acid sequences. J. Virol. **66:**1856–1865.
22. **Henderson, L. E., R. C. Sowder II, G. Smythers, and S. Oroszlan.** 1988. Chemical and immunological characterizations of equine infectious anemia virus *gag*-encoded proteins. J. Virol. **61:**2587–2595.
23. **Hicke, L., and H. Riezman.** 1996. Ubiquitination of a yeast plasma membrane receptor signals its ligand-stimulated endocytosis. Cell **84:**277–287.
24. **Hochstrasser, M.** 1995. Ubiquitin, proteasomes, and regulation of intracellular protein degradation. Curr. Opin. Cell Biol. **7:**215–223.
25. **Hochstrasser, M.** 1996. Protein degradation of regulation: Ub the judge. Cell **84:**813–815.
26. **Huang, M., J. M. Orenstein, M. Martin, and E. O. Freed.** 1995. $P6^{Gag}$ is required for particle production from full-length human immunodeficiency virus type 1 molecular clones expressing protease. J. Virol. **69:**6810–6818.
27. **Jentsch, S.** 1992. The ubiquitin-conjugation system. Annu. Rev. Genet. **26:**179–207.
28. **Levinger, L., and A. Varshavsky.** 1982. Selective arrangement of ubiquitinated and D1 protein-containing nucleosomes within the drosophila genome. Cell **28:**375–385.
29. **Loeb, K. R., and A. L. Haas.** 1992. The interferon-inducible 15-kDa ubiquitin homolog conjugates to intracellular proteins. J. Biol. Chem. **267:**7806–7813.
30. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **255:**265–275.
31. **Luftig, R. B., and L. D. Lupo.** 1994. Viral interactions with the host-cell cytoskeleton: the role of retroviral proteases. Trends Microbiol. **2:**178–182.
32. **Mueller, R. D., H. Yasuda, C. L. Hatch, W. M. Bonner, and E. M. Bradbury.** 1985. Identification of ubiquitinated histones 2A and 2B in Physarum polycephalum. J. Biol. Chem. **260:**5147–5153.
33. **Murti, K. G., H. T. Smith, and V. A. Fried.** 1988. Ubiquitin is a component of the microtubule network. Proc. Natl. Acad. Sci. USA **85:**3019–3023.
34. **Myers, G., J. A. Berzofsky, B. Korber, A. B. Rabson, and T. F. Smith.** 1990. Human retroviruses and AIDS 1990, a compilation and analysis of nucleic acid and amino acid sequences. Los Alamos National Laboratory, Los Alamos, N. Mex.
35. **Ott, D. E.** 1997. Cellular proteins in HIV. Rev. Med. Virol. **7:**167–180.
36. **Ott, D. E., L. V. Coren, D. G. Johnson, R. C. Sowder II, L. O. Arthur, and**

L. E. Henderson. 1995. Analysis and localization of cyclophilin A found in the virions of human immunodeficiency virus type 1 MN strain. AIDS Res. Hum. Retroviruses **11:**1003–1006.
37. **Ott, D. E., L. V. Coren, B. P. Kane, L. K. Busch, D. J. Johnson, R. C. Sowder II, E. N. Chertova, L. O. Arthur, and L. E. Henderson.** 1996. Cytoskeletal proteins inside human immunodeficiency virus type 1 virions. J. Virol. **70:** 7734–7743.
38. **Ott, D. E., S. M. Nigida, Jr., L. E. Henderson, and L. O. Arthur.** 1995. The majority of cells are superinfected in a cloned cell line that produces high levels of human immunodeficiency virus type 1 (MN). J. Virol. **69:**2443–2450.
39. **Parent, L. J., R. P. Bennett, R. C. Craven, T. D. Nelle, N. K. Krishna, J. B. Bowzard, C. B. Wilson, B. A. Puffer, R. C. Montelaro, and J. W. Wills.** 1995. Positionally independent and exchangeable late budding functions of the Rous sarcoma virus and human immunodeficiency virus Gag proteins. J. Virol. **69:**5455–5460.
40. **Putterman, D., R. B. Pepinsky, and V. M. Vogt.** 1990. Ubiquitin in avian leukosis virus particles. Virology **176:**633–637.
41. **Rey, O., J. Canon, and P. Krogstad.** 1996. HIV-1 Gag protein associates with F-actin present in microfilaments. Virology **220:**530–534.
42. **Sasaki, H., M. Nakamura, T. Ohno, Y. Matsuda, Y. Yuda, and Y. Nonomura.** 1995. Myosin-actin interaction plays an important role in human immunodeficiency virus type 1 release from host cells. Proc. Natl. Acad. Sci. USA **92:**2026–2030.
43. **West, M. H. P., and W. M. Bonner.** 1980. Histone 2B can be modified by the attachment of ubiquitin. Nucleic Acids Res. **8:**4671.