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Cytoskeletal Proteins inside Human Immunodeficiency Virus Type 1 Virions

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We have identified three types of cytoskeletal proteins inside human immunodeficiency virus type 1 (HIV-1) virions by analyzing subtilisin-digested particles. HIV-1 virions were digested with protease, and the treated particles were isolated by sucrose density centrifugation. This method removes both exterior viral proteins and proteins associated with microvesicles that contaminate virion preparations. Since the proteins inside the virion are protected from digestion by the viral lipid envelope, they can be isolated and analyzed after treatment. Experiments presented here demonstrated that this procedure removed more than 95% of the protein associated with microvesicles. Proteins in digested HIV-1_{MN} particles from infected H9 and CEM_{ss} cell lines were analyzed by high-pressure liquid chromatography, protein sequencing, and immunoblotting. The data revealed that three types of cytoskeletal proteins are present in virions at different concentrations relative to the molar level of Gag: actin (approximately 10 to 15%), ezrin and moesin (approximately 2%), and cofilin (approximately 2 to 10%). Our analysis of proteins within virus particles detected proteolytic fragments of α-smooth muscle actin and moesin that were cleaved at sites which might be recognized by HIV-1 protease. These cleavage products are not present in microvesicles from uninfected cells. Therefore, these processed proteins are most probably produced by HIV-1 protease digestion. The presence of these fragments, as well as the incorporation of a few specific cytoskeletal proteins into virions, suggests an active interaction between cytoskeletal and viral proteins.

The human immunodeficiency virus type 1 (HIV-1) virion is composed of both viral and host proteins. While the viral proteins have been studied since the discovery of the virus, host proteins that are specifically incorporated into the virion are just starting to be identified. Recently, the HLA class II-DR complex and cyclophilin A have been shown to be specifically incorporated into HIV-1 virions (3, 13, 33, 46). It may be possible to exploit both of these findings for antiviral therapies. The presence of HLA class II-DR complexes on the exterior of the virus might be used in the design of vaccines against HIV (3). Since cyclophilin A incorporation into HIV-1 virions is required for infectivity, compounds that block this association may become effective antiviral agents (13, 46). Other host proteins are likely to be incorporated into HIV-1 virions and might provide additional opportunities for antiviral therapies. Furthermore, the identification and characterization of these proteins are important for understanding the HIV-1 life cycle, virus-host interactions, and possibly HIV-1 pathology.

The study of host proteins inside HIV-1 virions is complicated by several factors. While the host proteins on the viral exterior can be studied by immunoprecipitation and neutralization of virions, these methods cannot be used to examine the interior proteins that are shielded from the outside by the viral membrane. A direct biochemical study of these proteins requires that the exterior proteins be removed so that only the proteins inside the virion remain. Therefore, these analyses are complicated by the presence of proteins that may contaminate virion preparations. One way that host proteins could contaminate purified virus preparations is by simply adhering to the virion exterior. A second and probably greater source of contamination is the presence of small membranous particles, termed microvesicles, that are released by activated cells (2, 48). These particles contain considerable amounts of host proteins. A significant fraction of microvesicles copurify with HIV-1 virions by sucrose density centrifugation and have been observed in electron micrographs of HIV-1 preparations (5). Therefore, proteins from either of these sources are likely to contaminate purified virion preparations. Thus, proteins from both of these sources must be eliminated to clearly identify and characterize the host proteins inside the virion.

To remove these sources of contamination, we have developed a method for digestion of virus preparations with subtilisin. The treatment removes proteins on the exterior of the virion and eliminates nearly all of the microvesicles in purified HIV-1 preparations (33). In contrast, the interior proteins, both viral and host, are protected from digestion by the lipid envelope and can be analyzed.

Cytoskeletal proteins, because of their proximity to the viral assembly and budding sites, may be incorporated inside HIV-1 virions. Actin has been found in preparations from several types of retroviruses; these observations have led to the suggestion that the cytoskeleton is involved in the assembly and budding of retroviruses (9, 50). The presence of actin in highly purified HIV-1_{MN} virion preparations (3) also supports this possibility. However, actin and other cytoskeletal proteins are highly abundant proteins and are readily found in tissue culture supernatants of uninfected cells (5, 6). Therefore, the presence of cytoskeletal proteins in virus preparations can only suggest and not prove their presence inside the virion.

We have used the digestion method to analyze HIV-1_{MN} virions for cytoskeletal proteins by high-pressure liquid chromatography (HPLC), immunoblotting, and amino acid se-

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quencing methods. Our results showed that the treatment removes more than 95% of the actin that copurifies with virions in viral preparations. The analyses indicated that several components of the cytoskeletal network, i.e., nonmuscle and muscle actins, cofilin, ezrin, and moesin, are present inside the virions. Additionally, actin and moesin fragments that were cleaved at consensus HIV-1 protease sites were found inside virions. The presence of these proteins and proteolytic fragments inside virions suggests an interaction between HIV-1 and the cytoskeleton.

MATERIALS AND METHODS

Virus preparations and digestions. Cell lines were cultured in RPMI 1640 containing 10% fetal bovine serum, 2 mM glutamine, 100 U of penicillin per ml, 100 μg of streptomycin per ml (Life Technologies, Gaithersburg, Md.). HIV-1_{MN} in tissue culture medium from Clone 4 (an HIV-1_{MN}-infected cell clone of H9 [34]) and infected CEM_{ss} cells was purified twice by sucrose density gradient centrifugation as previously described (6). Medium from uninfected cells was treated by the same method as for virions, except that only one sucrose density purification step was used to isolate the microvesicles and other cellular material that bands at 1.16 g/ml (5). The resulting preparations are referred to as mock virus (5). Virus from HIV-1-infected SupT1 and SupT2 cells and peripheral blood mononuclear cells was a kind gift of David Waters (AIDS Vaccine Program, National Cancer Institute—Frederick Cancer Research and Development Center, Frederick, Md.). For these preparations, virus from clarified tissue culture supernatants was pelleted, gently resuspended in TE (20 mM Tris-HCl [pH 8.0], 2 mM EDTA), and banded in a 30 to 45% sucrose density step gradient. Virion digestions were carried out as previously described (33), using 1-mg/ml subtilisin with virion preparation protein concentrations ranging from 2 to 4 mg/ml as assayed by the method of Lowry et al. (25). Virions from digested preparations were then repurified by centrifugation through 20% sucrose and resuspended in Dulbecco's phosphate-buffered saline without $CaCl_2$ or $MgCl_2$ (Life Technologies Inc.) or in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) or HPLC sample loading buffers as appropriate for the intended analysis.

Protein analysis. Immunoblot analysis was carried out as previously described (17) by using the enhanced chemiluminescence (ECL) antibody detection system (Amersham Lifescience Inc., Arlington Heights, Ill.) and exposure to Lumi-Film (Boehringer Mannheim, Indianapolis, Ind.). The mouse monoclonal antibodies used were as follows: anti-α-smooth muscle actin (43) and anti-α-sarcomeric actin (Accurate, Westbury, N.Y.); anti-β-actin (Sigma, St. Louis, Mo.) (31); anti-pan-actin (Amersham); and anti-ezrin (Zymed Laboratories, Inc., San Francisco, Calif.). Goat $p24^{CA}$ antiserum was produced by the AIDS Vaccine Program, National Cancer Institute—Frederick Cancer Research and Development Center. Immunoblots were stripped of antibody complexes to allow for reprobing by incubating the membrane filters in stripping buffer (100 mM β-mercaptoethanol, 2% [wt/vol] SDS, 62.5 mM Tris-HCl [pH 6.7]) at either 50 or 60°C for 30 min with agitation and then giving them two 10-min washes in TBS (20 mM Tris-HCl [pH 7.6], 137 mM NaCl) with 0.1% Tween 20 (Sigma). The filters were then blocked for 2 h and reacted by using the above-mentioned ECL protocol. Amino acid sequence analysis and reverse-phase HPLC were performed as previously described (16).

RESULTS

Actin isoforms in HIV-1 preparations. Six major isoforms of actin are found in mammalian cells. The amino acid sequences of these isoforms are nearly identical, varying primarily within the N-terminal 20 residues (49). Sequence, localization, and function data have allowed for the classification of actin isoforms into two groups: muscle and nonmuscle actins (reviewed in reference 18). Muscle actins consist of α-skeletal, α-cardiac, and γ-smooth muscle actin that are found mostly in striated muscle, cardiac muscle, and smooth muscle cells, respectively, while α-smooth muscle actin is found in both muscle and nonmuscle cells. β- and γ-nonmuscle actins are found in a wide variety of nonmuscle cell types.

Previous studies have shown that the relative expression of actin isoforms in lymphoid cells changes upon activation of the cells (24). Since actin is present in highly purified HIV-1 preparations (3), the presence of α-smooth muscle and β-actin isoforms in HIV-1 virions produced from several different cell lines was determined by immunoblot analysis. Additionally, the presence of these isoforms in mock-virus preparations (tissue culture supernatants from uninfected cells that were purified in the same manner as the purified virion preparations [6]) was determined by immunoblot analysis. The results of probing duplicate blots with monoclonal antibodies specific for either α-smooth muscle or β-actin isoforms revealed that only one isoform was detected in the preparations tested (Fig. 1). The lanes containing both H9 mock virus and virus preparations purified from Clone 4 culture supernatants stained with α-smooth muscle actin antibody and had no detectable β-actin antibody staining. In contrast, the lanes that contained HIV-1 preparations produced from HIV-1-infected CEM_{ss}, SupT1, and SupT2 cells and primary peripheral blood mononuclear cells, as well as CEM_{ss} mock-virus preparations, all stained with the β-actin antibody with no detectable α-smooth muscle actin antibody staining. Additional immunoblot analyses determined that α-skeletal actin was not present in any of the virion preparations (data not shown). The finding that uninfected cells release actin which purifies under the same conditions as the virions suggests that the virus preparations might also be contaminated with actin. The buoyant density of this actin indicates that it is associated with dense particles (5), most probably microvesicles that are released from cells (2, 48). In light of this result, the analysis of cytoskeletal proteins that are inside the virion requires the removal of the contaminating proteins associated with microvesicles.

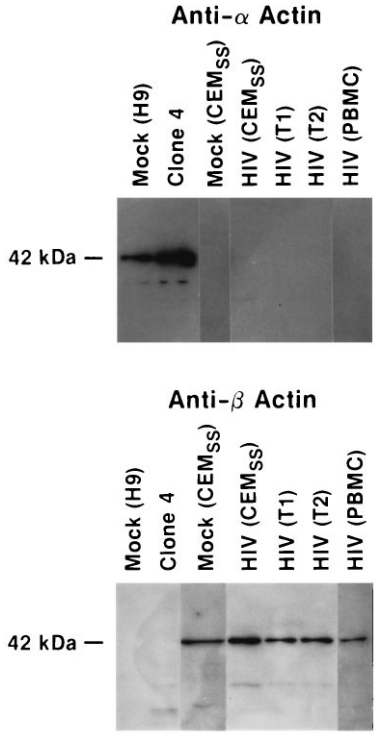

FIG. 1. Actin immunoblot analysis of virus and mock-virus preparations. Duplicate blots of virus and mock-virus preparations isolated and purified from tissue culture supernatants were reacted with antibody specific for either α-smooth muscle actin or β-actin as denoted above the blots.

Actin inside the virion. Recently, we have described a protease digestion method that eliminates the proteins on the exterior of the virion (33). In addition to removing the exterior proteins, electron microscopic analysis of treated virus preparations demonstrated that the method also eliminated nearly all of the contaminating microvesicles (33). This digestion and centrifugation method was used to determine if actin is present

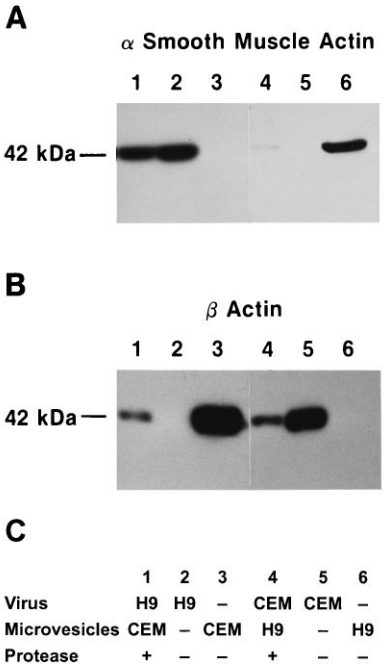

FIG. 2. Immunoblot analysis of digested virus-microvesicle mixture. (A and B) Duplicate blots of mixed virion and microvesicle preparations (10 μg of each protein) that were digested and 10 μg of protein for the respective untreated controls were reacted with antibody specific for α-smooth muscle actin (A) or β-actin (B). Lanes: 1, digested and density centrifuged virions from Clone 4 cells mixed with microvesicles from CEM_{ss} cells; 2, untreated virions from Clone 4 cells; 3, untreated microvesicles from CEM_{ss} cells; 4, digested and density centrifuged virions from CEM_{ss} cells mixed with microvesicles from H9 cells; 5, untreated virions from CEM_{ss} cells; 6, untreated microvesicles from H9 cells. (C) Summary of the materials and treatment for each lane.

inside the virion. Additionally, the efficiency of microvesicle removal was monitored by adding an equal amount of microvesicles to the virion preparation before digestion. To distinguish between virus- and microvesicle-associated actin, the seeded microvesicles were produced from a cell line that incorporates a different isoform into the particles from that in the cell line used to produce the virions (e.g., mock virus produced from H9 cells added to virus produced from CEM_{ss} cells). The amount of actin remaining after digestion from either the virions or the microvesicles was detected by immunoblot analysis for the unique actin isoform present in each type of particle. In this way, the separate fates of virion and microvesicle actin after treatment can be monitored in the same experiment.

Virions produced from Clone 4 cells (reacting solely with α-smooth muscle actin antibody) were mixed with an equal amount of total protein from a CEM_{ss} microvesicle preparation (reacting solely with β-actin antibody). After digestion and density centrifugation, the remaining virion-associated actin in the mixture was detected by an α-smooth muscle actin immunoblot. The blot revealed that the amount of α-smooth muscle actin present in the treated sample was slightly reduced (Fig. 2A, lane 1) compared with an equivalent amount of untreated Clone 4 virion preparation (lane 2). The blot was stripped and reacted with $p24^{CA}$ antiserum to determine if the internal proteins were protected. The results showed equivalent staining intensity in the treated and untreated lanes (data not shown), consistent with our previous results (33) that the interior of the virion is protected after treatment. The lane containing untreated CEM_{ss} microvesicles (lane 3) had no reactivity, showing that the added microvesicles did not contribute to the α-smooth muscle actin antibody staining. The microvesicle-associated actin remaining in the treated mixture was examined by staining a duplicate blot with β-actin-specific antibody (Fig. 2B, lane 1). The results showed that the β-actin signal from the lane containing the treated mixture was considerably weaker than that of the lane containing an equal amount of untreated CEM_{ss} microvesicles (lane 3). The Clone 4 virus lane (lane 2) had no reactivity with the antibody, showing that the virions did not contribute any β-actin signal to these results. Taken together, these data show that microvesicle-associated actin was largely removed by this procedure, even though nearly all of the actin associated with virions remained (Fig. 2A, lanes 1 and 2). Since most of the actin was protected from the protease treatment, it is present inside the virion.

To confirm the removal of microvesicles by this method, the reciprocal mixture of virions produced from CEM_{ss} cells (reacting solely with the β-actin antibody) and microvesicles produced from H9 cells (reacting solely with α-smooth muscle actin antibody) was treated. The results of the immunoblot analysis for microvesicle-associated actin were determined by α-smooth muscle actin staining. Similar to the results described above, the treated mixture sample (Fig. 2A, lane 4) had greatly reduced amounts of α-smooth muscle actin compared with the sample containing an equal amount of untreated H9 microvesicles (lane 6). Consistent with the results of Fig. 1, the CEM_{ss} virions (Fig. 2A, lane 5) did not react with α-smooth muscle actin. Taken together, the data from both experiments clearly show that treatment by protease digestion and sucrose density centrifugation eliminated most of the microvesicle-associated actin. From immunoblot experiments on these digested mixtures and dilution series of appropriate microvesicle preparations, we estimate that more than 95% of the microvesicle-associated actin is eliminated by this procedure (data not shown).

The amount of actin remaining from the CEM_{ss} virion preparation was determined by β-actin immunoblot analysis. Unlike the data for Clone 4 virions, the results showed that the staining intensity in the treated-mixture sample (Fig. 2B, lane 4) was considerably weaker than that in the untreated CEM_{ss} virion preparation control lane (lane 5). However, stripping and probing this immunoblot with $p24^{CA}$ antibody detected no reduction in staining intensity in these two lanes, showing that the proteins inside the virus were not digested (data not shown). Therefore, only a fraction of the actin in the CEM_{ss} preparation was present inside these virions. Since preparations from CEM_{ss} have considerable amounts of microvesicles (5), the actin that was lost to digestion was most probably associated with microvesicles that were removed by this method. In contrast, the Clone 4 cell line produces virus preparations that contain relatively few microvesicles (5, 33); thus, the amount of signal loss after treatment was negligible for these preparations.

HPLC analysis of actin inside HIV-1 virions produced from H9 cells. The actin present in virions was examined by HPLC, immunoblot, and sequence analysis of digested $HIV\text{-}1_{MN}$ isolated from Clone 4 cells. A 25-mg portion of the virion preparation was digested by subtilisin, and the digested virions were isolated by sucrose density centrifugation to remove residual protein fragments. Samples (1 mg) of the preparation both before and after treatment were analyzed on an analytical-scale HPLC column, and the fractions were subjected to SDS-PAGE analysis and Coomassie blue staining. The chromatograms showed that while equivalent levels of Gag proteins were present for both untreated and treated samples, all of the $gp120^{SU}$ peak and many of the peaks associated with host

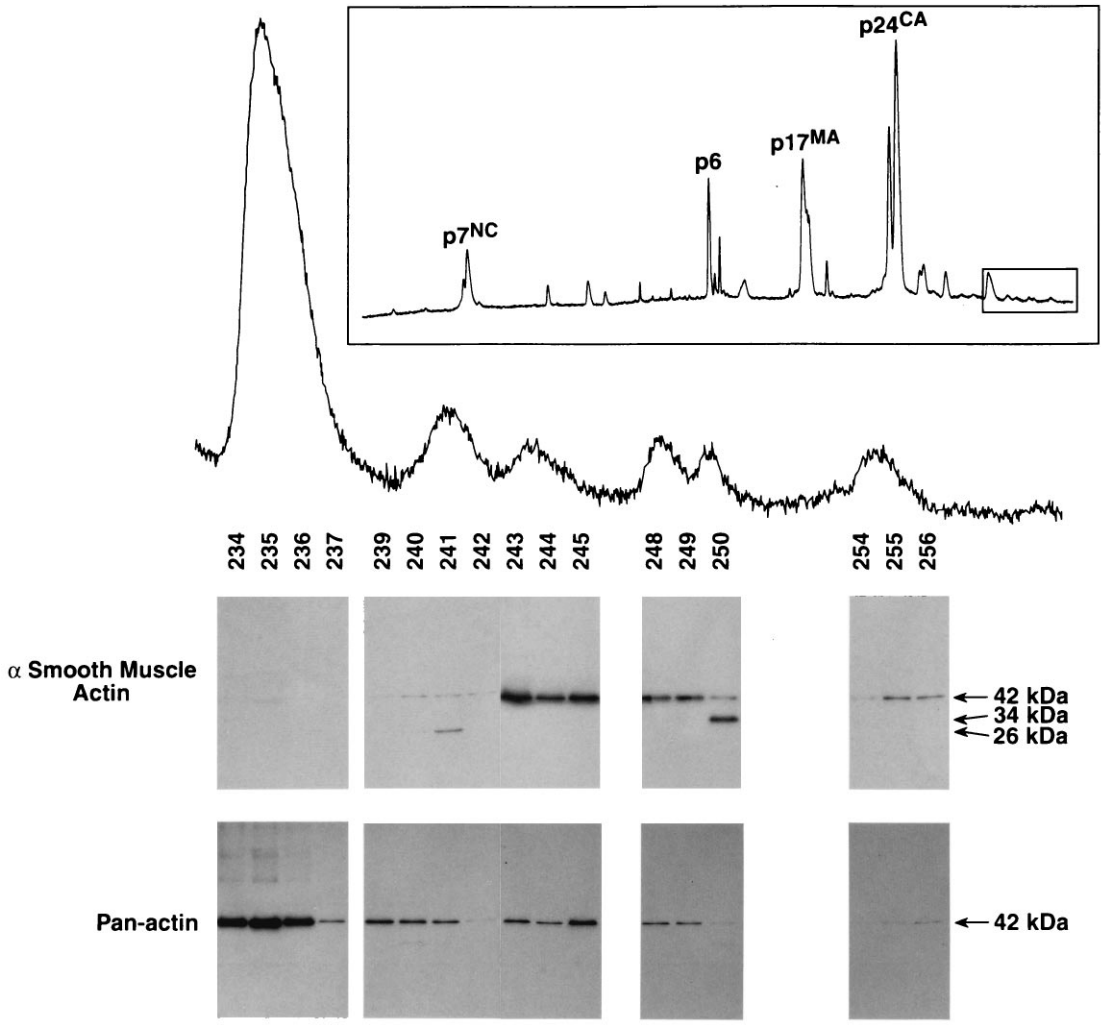

FIG. 3. HPLC and immunoblot analysis of digested virions produced from Clone 4 cells. The actin-containing region of the HPLC (highlighted by a box in the inset showing the complete HPLC profile) is presented. Immunoblot analysis of selected fractions with antibody for either α-smooth muscle actin or pan-actin is displayed under the respective peaks on the HPLC figure and identified by the fraction number. The sizes of the bands, determined by relative mobility, are indicated to the right of the blots.

proteins were significantly reduced in the treated samples (data not shown). This confirms that the method removes the exterior proteins while leaving the interior proteins intact. The remaining 24 mg of digested virions was then applied to a preparative HPLC column, and the collected fractions were analyzed for actin by SDS-PAGE analysis and Coomassie blue staining as well as immunoblot analysis.

Immunoblot analysis of actin-containing HPLC fractions for α-smooth muscle actin and pan-actin antibody reactivity is presented in Fig. 3. No appreciable β-actin staining was detected in these fractions, as shown above (Fig. 1 and 2). Staining with antibody specific for α-smooth muscle actin revealed that the majority of this protein was contained in a broad peak covering fractions 243 to 245 that tails off through fraction 250. While most of the α-smooth muscle actin in this preparation was the full 42 kDa, a smaller amount of truncated 26- and 34-kDa species was also detected in fractions 241 and 250, respectively. These fragments are the N-terminal portions, since this α-smooth muscle actin antibody reacts with the first 10 N-terminal residues of this isoform (43).

Previous studies by Tomasselli et al. have demonstrated that HIV-1 protease can cleave α-smooth muscle actin after amino acid 270 in vitro (47). Cleavage at this site would produce an N-terminal fragment with a calculated mass of 34.6 kDa; this matches the 34-kDa band observed by immunoblotting with the α-smooth muscle actin antibody (Fig. 3). To verify this possibility, the corresponding 8-kDa C-terminal cleavage product was isolated (present just after $p17^{MA}$, between the two cyclophilin peaks) and identified by HPLC and sequence analysis of an undigested Clone 4 virus preparation. The sequence analysis showed that this fragment was a cleavage product of α-smooth muscle actin that starts at residue 271 (Fig. 4A). These data confirm that α-smooth muscle actin in these virions is cleaved at the HIV-1 protease site after amino acid 270.

The 26-kDa band in the α-smooth muscle actin immunoblot that is present in the HPLC fraction 241 (Fig. 3) may represent another N-terminal fragment produced by HIV-1 protease cleavage. However, the C-terminal half of this species has not been identified in the HPLC fractions, possibly because of the small amount of this fragment in the virions. Without the sequence of the corresponding C-terminal fragment, the origin of the 26-kDa fragment is not clear. Analysis of H9 mi-

A

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                261     > 271                                                 340
8 kDa Fragment          MESAGIHETT YNSIMKCDID IRKDLYANNV LSGGTTMYPG IADRMQKEIT
α-Smooth Muscle ETLFQPSFIG ---------- ---------- ---------- ---------- ----------
γ-Smooth Muscle ETLFQPSFIG ---------- ---------- ---------- ---------- ----------
β-Nonmuscle     EALFQPSFLG ---C------ F--------V- ---------T- ---------- ----------
γ-Nonmuscle     EALFQPSFLG ---C------ F--------V- ---------T- ---------- ----------

                341                                                     377
8 kDa Fragment  ALAPSTMKIK IIAPPERKYS VWIGGSILAS LSTFQQMWIS KQEYDEAGPS IVHRKCF
α-Smooth Muscle ---------- ---------- ---------- ---------- ---------- -------
γ-Smooth Muscle ---------- ---------- ---------- ---------- -P-------- -------
β-Nonmuscle     ---------- ---------- ---------- ---------- ------S--- -------
γ-Nonmuscle     ---------- ---------- ---------- ---------- ------S--- -------
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B

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                Peptide 1                          Peptide 2
                87                  107            121                 146
Lys-C Peptide   IWHHTFYNELRVAPEEHPVLL              MTQIMFETFNTPAMYVAIQAVLSLYA
β-Nonmuscle     ---------------------              -------------------------
γ-Nonmuscle     ---------------------              -------------------------
γ-Smooth Muscle ----S-------------T--              ----------V--------------
α-Smooth Muscle ----S-------------T--              ----------V--------------

                Peptide 3                          Peptide 4
                194                 215            241                 270
Lys-C Peptide   ILTERGYSFTTTAEREIVRDIK              SYELPDGQVITIGNERFRCPEALFQPSFLG
β-Nonmuscle     ----------------------             ------------------------------
γ-Nonmuscle     ----------------------             ------------------------------
γ-Smooth Muscle ---------V------------             ----------------------T------I-
α-Smooth Muscle ---------V------------             ----------------------T------I-
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FIG. 4. Sequence comparison of actins from Clone 4 and nonskeletal isoforms. (A) The 8-kDa protein sequence (highlighted by an arrowhead) is shown on the top line in the single-letter amino acid code and compared with the published human α-smooth muscle actin (20), γ-smooth muscle actin (27), γ-nonmuscle actin (12), and β-actin (38) sequences. Identical amino acids are indicated by a dash, and different amino acids are denoted by the single-letter amino acid code. (B) Amino acid sequences of four peptides obtained from the Lys-C digestion of actin from HPLC fractions 234 to 237 (Fig. 3) of Clone 4-produced virus are presented. The sequences of the Lys-C-generated peptides are shown on the top line and are compared with the corresponding regions of β-actin (38), γ-nonmuscle actin (12), γ-smooth muscle actin (27), and α-smooth muscle actin (20) sequences. Identical amino acids are shown by dashes, and amino acid differences are denoted by the single-letter code.

crovesicle preparations has not detected either the 26- or the 34-kDa fragments of α-smooth muscle actin and demonstrates that these products are specific to virion preparations (data not shown).

The α-smooth muscle actin antibody reacts with the fractions from only one of the peaks that contain a 42-kDa protein by SDS-PAGE analysis and Coomassie blue staining. Immunoblot analysis of HPLC fractions in this region with an antibody that detects all isoforms of actin (pan-actin) was performed and revealed an antibody staining peak covering fractions 234 to 236 that did not react with the α-smooth muscle actin antibody (Fig. 3). Since it is often found in nonmuscle cells, the γ-nonmuscle isoform of actin is a likely candidate for this other actin peak. Clear identification of this isoform is difficult, since this actin is immunologically similar to the other actins and we have yet to obtain a γ-nonmuscle actin-specific antibody. Since actin molecules are blocked in N-terminal sequencing by amino acetylation (49), protein sequencing was performed on peptides generated by Lys-C protease digestion of this actin. Four peptides spanning actin residues 87 to 107, 121 to 146, 194 to 215, and 241 to 270 were sequenced in regions that have amino acid differences among the human isoforms (12, 20, 27, 38). The alignment of these peptide sequences with human actin sequences showed that the actin in fractions 234 to 236 was either β- or γ-nonmuscle actin (Fig. 4B). These two isoforms differ only in the first 10 amino acids which are blocked to N-terminal sequence analysis (12, 38, 49). Since antibody specific for the N terminus of β-actin did not react with Clone 4 viral preparations (Fig. 1 and 2B) or with this peak (data not shown), this protein is most probably γ-nonmuscle actin by virtue of both the immunoblot and sequence data.

The HPLC data allow for an estimation of the amount of actin in virions produced by Clone 4: γ-nonmuscle actin is the major isoform over the α-smooth muscle isoform at a molar ratio of 5:1. Overall, we estimate (from the relative abundance of $p17^{MA}$ to the total actin) that actin is present at approximately 10% of the molar level of Gag.

HPLC analysis of HIV-1 produced from CEM_{ss} cells. The actin present in virions produced by infected CEM_{ss} cells was also analyzed by HPLC, SDS-PAGE and Coomassie blue staining, and immunoblot analysis. A 50-mg sample of the virion preparation was treated with protease, and the digested virions were isolated by density centrifugation. Samples (2 mg) of both treated and untreated virions were analyzed by analytical HPLC, which confirmed the loss of exterior proteins and the maintenance of the interior viral proteins (data not shown). The remaining 48 mg was subjected to preparative HPLC. SDS-PAGE analysis and Coomassie blue staining located the HPLC fractions that contain a 42-kDa protein. Immunoblot

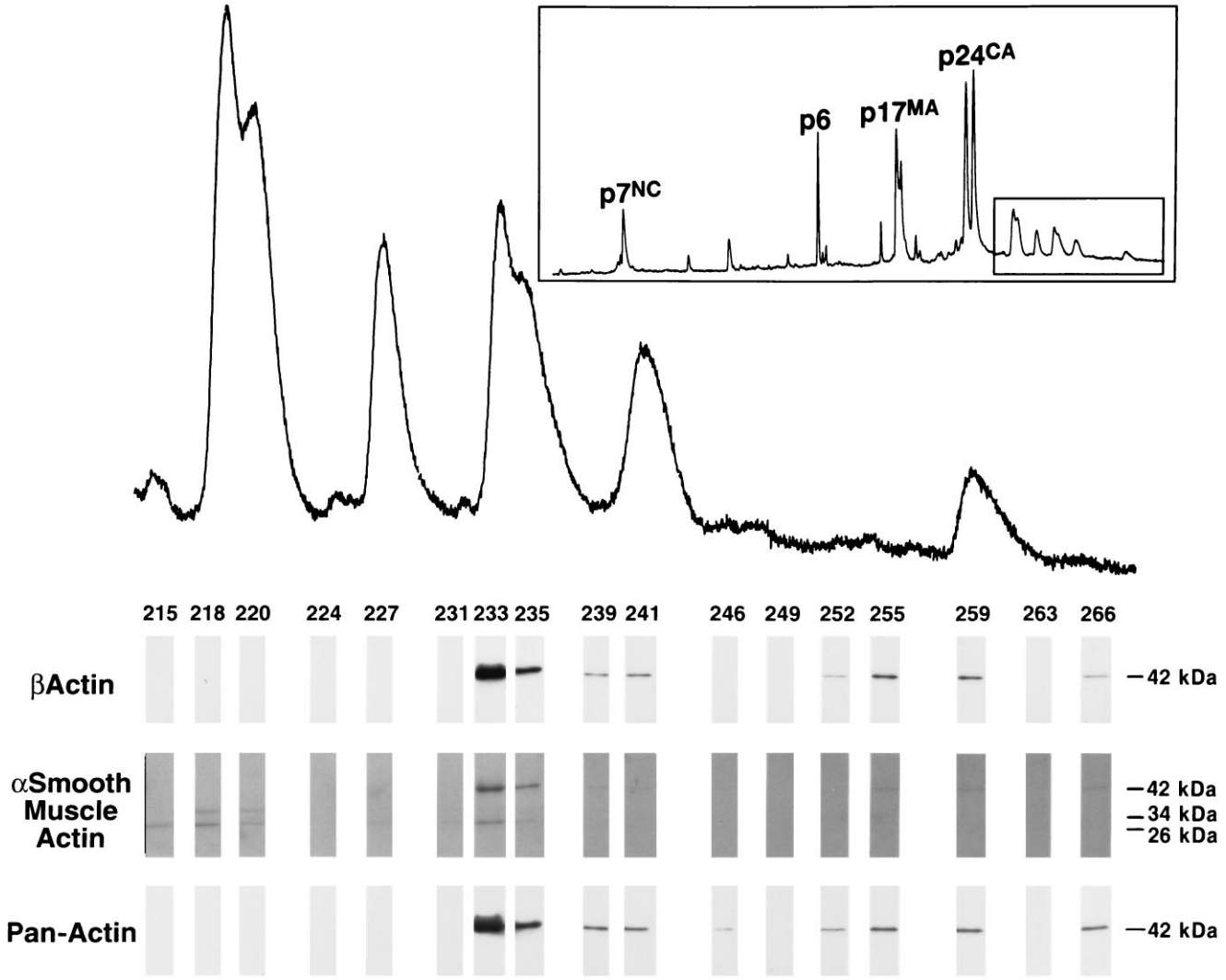
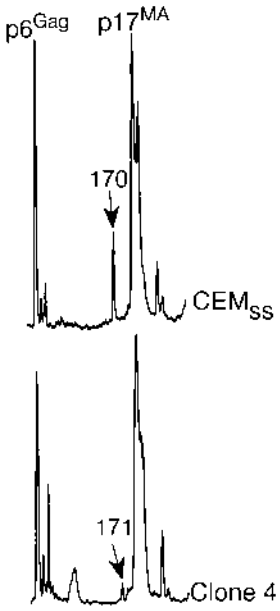


FIG. 5. HPLC and immunoblot analysis of digested virions produced from CEM_{ss} cells. The actin-containing region of the HPLC (highlighted by a box in the inset showing the complete HPLC profile) is presented. Immunoblots of selected fractions with β-actin, α-smooth muscle actin, or pan-actin antibody are displayed under the respective peaks and identified by the fraction number. The sizes of the bands, determined by relative mobility, are indicated to the right of the blots.

analysis of these HPLC fractions for β-actin detected staining in a major peak (fractions 233 to 235) that tails out to fraction 241 (Fig. 5). Some β-actin that is not associated with a definite peak was also detected at fractions 253 to 259. Analysis of the same fractions by α-smooth muscle actin immunoblotting revealed that a small but readily detectable amount of this actin was present in fractions 233 to 235 as well as 26- and 34-kDa N-terminal protein fragments (Fig. 5). These smaller bands are likely to be similar to the 26- and 34-kDa bands seen in the Clone 4 preparation. A corresponding C-terminal 8-kDa fragment of α-smooth muscle actin has not been found in these HPLC fractions because of the low levels of α-smooth muscle actin in the CEM_{ss} virions. The immunoblot stained with the pan-actin antibody detected a nearly identical staining intensity across the fractions to that in the β-actin blot, indicating that most of the actin present in these fractions is the β isoform. Thus, while a minor amount of γ-nonmuscle actin may be present in this peak, the major isoform of actin present in CEM_{ss}-produced virus is β-actin.

From the HPLC data, we estimate that β-actin (with possibly some γ-nonmuscle actin) makes up more than 98% of the actin inside the CEM_{ss}-produced virions, with only a minor amount of α-smooth muscle actin present. In total, these virions have actin at 15% of the molar Gag levels.

Presence of actin-binding proteins in HIV-1_{MN}. While actin is the major component of the cytoskeleton, other cytoskeletal proteins are associated with actin. These proteins bind and regulate the polymerization of actin, while other proteins cross-link actin filaments to form lattices and fibers as well as to link the cytoskeleton to the plasma membrane (37). HPLC and SDS-PAGE analysis revealed the presence of a 21-kDa protein in the virions produced by CEM_{ss} cells in fraction 170 (Fig. 6). Attempts at protein sequence analysis indicated that the amino terminus of this protein was blocked. To obtain internal sequences, the isolated 21-kDa protein was digested with Arg-C protease and fragments were purified by HPLC purification. Two of the fragments were sequenced. This analysis produced two sequence stretches that exactly match two sequences (residues 33 to 49 and 82 to 93) found in cofilin, an actin-binding and -depolymerizing protein (Fig. 6) (32). SDS-



Fraction 170: KKAVLFXLSEDXXNIIL
Cofilin 33-49: KKAVLFCLSEDKKNIIL

Fraction 170: YALYDATYETK
Cofilin 82-93: YALYDATYETK

FIG. 6. HPLC and protein sequence analysis of cofilin. Selected HPLC regions of both the CEM_{ss} and Clone 4 virion samples are shown, with the peaks in fractions 170 and 171 labeled. The amino acid sequences of the two peptides are presented under the chromatograms and compared with the corresponding stretches of cofilin (32). Residues are denoted by the single-letter amino acid code, and X denotes positions in the sequence where the yield of amino acid recovered was insufficient to make a definitive assignment.

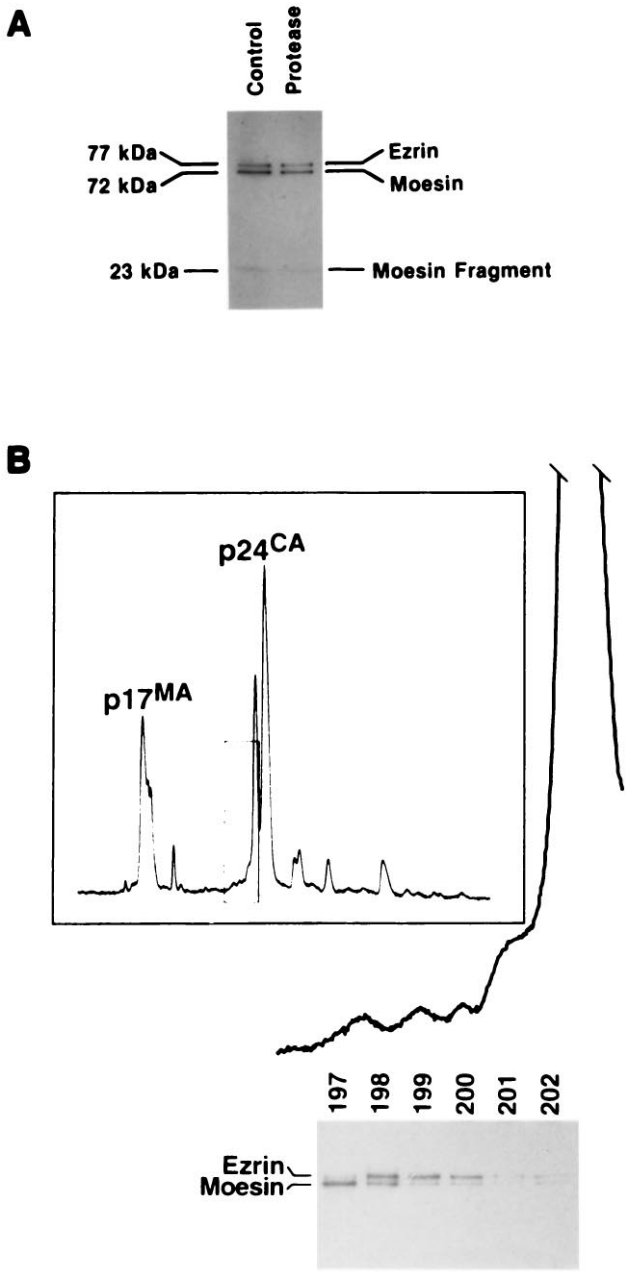


FIG. 7. Analysis of Clone 4 virions for ezrin and moesin. (A) Virions incubated with buffer (Control) or subtilisin (Protease) were immunoblotted and reacted with antibody against ezrin. (B) HPLC and immunoblot analysis of treated virions. The ezrin- and moesin-containing region of the HPLC (highlighted by a box in the inset showing a portion of the HPLC profile between $p17^{MA}$ and $p24^{CA}$) is presented. Fractions selected for immunoblot analysis with ezrin antibody are labeled above the blot.

PAGE analysis of the same region from the HPLC analysis of the Clone 4-produced virions detected only a small amount of a 21-kDa protein in fraction 171 that was insufficient for digestion and sequencing (data not shown). Examination of the elution profile along with the SDS-PAGE and Coomassie blue staining results shows that approximately one-half of this peak in the Clone 4 chromatograph is likely to consist of the 21-kDa cofilin protein. From the HPLC data, the cofilin inside virions from the CEM_{ss} preparation was present at approximately 11% of the level of molar Gag while the virions from Clone 4 contained approximately 2% of the level of molar Gag.

In addition to cofilin, our HPLC, sequence, and immunoblot analysis of HIV-1_{MN} preparations have found ezrin and moesin in virions. These two proteins cross-link actin and the plasma membrane (1, 4, 7, 15, 23, 35). Ezrin and moesin are closely related, and the monoclonal antibody used in this study cross-reacts with a shared epitope in the C terminus of these proteins (data not shown). Immunoblot analysis (Fig. 7A) with anti-ezrin antibody demonstrated that subtilisin treatment of virions produced by Clone 4 did not significantly reduce the amount of the 77-kDa ezrin band and the cross-reacting 72-kDa moesin signals in the digested lane compared with the control lane. The sizes of both of these proteins as determined by relative mobility are somewhat smaller than the published sizes of 80 kDa for ezrin (7) and 77 kDa for moesin (4). This is likely to be due to technical differences and not proteolysis, since the electrophoretic mobilities of ezrin and moesin from microvesicles and virions are the same (data not shown). Similar results were obtained with CEM_{ss}-produced virions. HPLC fractionation of digested virions from Clone 4 cells followed by immunoblot analysis found that ezrin and moesin eluted just before $p24^{CA}$ in two coeluting bands, with fraction 197 containing the largest amount of moesin while fractions 198 to 200 contained the majority of the ezrin (Fig. 7B). Thus, our anal-

yses show that both ezrin and moesin are incorporated into HIV-1 virions. Virions produced from CEM_{ss} cells contained similar levels of full-length ezrin and moesin at levels of approximately 2% of that of molar Gag.

In addition to full-length ezrin and moesin, the anti-ezrin immunoblot analysis (Fig. 7A) detected a 23-kDa fragment in both the control and treated virion samples. The presence of this fragment in both the untreated and treated samples shows that it was not produced by the subtilisin digestion. This fragment may be a cleavage product of either ezrin or moesin, since the antibody reacts with both proteins. HPLC analysis of virions produced by both Clone 4 and CEM_{ss} cells was used to isolate this 23-kDa fragment, which eluted at fraction 123 of the HPLC, earlier in the profile than the full-length ezrin and moesin proteins. N-terminal sequencing of this protein revealed that the molecule was a C-terminal cleavage product of moesin with an N terminus that started with moesin residue 451. The P_1, P_1', P_2', P_3', and P_4' residues that flank the cleavage site (KAQM–451-VQED) in full-length moesin are consistent with an HIV-1 protease cleavage site (39), although in vitro cleavage at this site has not been reported. This fragment appears to be virus specific, because microvesicle preparations do not contain detectable fragments of either moesin or ezrin. Therefore, similar to α-smooth muscle actin, moesin may be cleaved by HIV-1 protease.

DISCUSSION

We have developed a digestion method that removes proteins on the exterior of the virion so that the proteins inside HIV-1 virions can be studied (33). In this report, we demonstrate that this method also effectively removes microvesicles (eliminating more than 95% of the microvesicle-associated actin in virion preparations). This result supports our previous conclusions from electron microscopic data that nearly all of these particles are removed by this treatment (33). The elimination of microvesicles probably results from a reduction in the density of these particles after protease digestion that allows these "lighter microvesicles" to be separated from the heavier virions by density centrifugation. Analyses of digested microvesicles isolated from virion and from microvesicle preparations have shown that they contain negligible amounts of intact proteins (8). Thus, unlike the viral membrane, the microvesicle membrane does not appear to protect the proteins inside.

Taken together, our results demonstrate that digested virion preparations are a good source of material to use in the study of proteins inside virions. Using this technique along with biochemical analysis on $HIV\text{-}1_{MN}$ virions, we have found that several cytoskeletal proteins, i.e., actin, cofilin, ezrin, and moesin, are present in $HIV\text{-}1_{MN}$ virions.

HPLC and immunoblot analysis of virions produced from Clone 4 (H9) and CEM_{ss} cells demonstrated that the actin isoforms present inside the particle varied with the cell line used to produce the virus. Virions produced from Clone 4 cells contained mostly γ-nonmuscle actin (approximately 80%) with some α-smooth muscle actin (approximately 20%). In virions produced by CEM_{ss} cells, the bulk of the actin (more than 98%) present was the β-actin isoform (although a small amount of γ-nonmuscle actin also may be present) with only a trace amount of α-smooth muscle actin (less than 2%). While the amount of actin lost by digestion depended on the cell line used for virus production, the majority of the actin was present in both viral preparations inside the particle. The amount of actin inside the virions ranged from 10 to 15% of molar Gag levels for virus produced from Clone 4 and CEM_{ss} cells, respectively.

The presence of a cytoskeletal protein in the virion may indicate which cellular structures interacted with the assembling and budding virion in the cell. Nonmuscle actins are associated mostly with the cortex of the plasma membrane (9, 10, 18) and function primarily by shaping the plasma membrane. They also produce specialized structures such as pseudopods and microvilli (28). Our data show that these cortical actins, either the β- or γ-nonmuscle isoforms, are present inside the virion and suggest that an interaction between the virion and the cortical cytoskeleton occurs during assembly and budding. The differences in actin isoform patterns for H9 and CEM_{ss} preparations probably reflect the makeup of the cortical cytoskeleton in each cell line, because both microvesicles and virions contain similar distributions of actin. It has been shown that resting lymphocytes generally express mostly β-actin and little, if any, γ-nonmuscle actin, consistent with our data for CEM_{ss}-produced particles (24). However, activated lymphocytes were shown to have increased levels of γ-nonmuscle actin, similar to our data for H9-produced particles (24).

While the isotypes of the cortical actin inside the virion varied with the producer cell line, both types of virions contained α-smooth muscle actin. Despite its name, this isoform is also found in nonmuscle cells, predominantly associated with microfilaments that connect the interior of the cell with the cortical actin network (18, 19, 42). Therefore, it is possible that the nascent HIV-1 virions interact with microfilaments during assembly and budding. This possibility is consistent with two studies of murine leukemia virus Gag and the cytoskeleton. In one study, approximately 10% of a temperature-sensitive murine leukemia virus Gag mutant was associated with the actin cytoskeleton (11). Results from immunofluorescence microscopy studies with cells producing murine leukemia virus have also shown an association of Gag and Gag-Pol precursors with microfilaments inside infected cells (26). Recently, Rey et al. have demonstrated the association of HIV-1 Gag with the actin cytoskeleton in infected cells by immunological and biochemical methods (39a). An interaction between HIV-1 and α-smooth muscle actin is also supported by the detection of HIV-1 protease cleavage fragments of α-smooth muscle actin inside virions produced from both cell types. Recent reports have shown that protease is activated before the completion of budding and may be required for efficient release from the cell (21, 22). Therefore, the cleavage of α-smooth muscle actin may occur during the budding process and might be required for the efficient release of HIV-1 virions. However, protease cleavage also could occur after the assembly and budding process.

Several other components of the cytoskeleton were also found to be present in virions. While actin self-assembles into filaments, the formation of filaments is regulated by actin-binding proteins that sequester monomeric actin, block filament formation, or depolymerize the filaments directly (37). Our results determined that one of these proteins, cofilin, was present in both types of virions, although virions produced by CEM_{ss} cells contained considerably more cofilin (at 10% of the molar Gag level) than did those from Clone 4 cells (at 2% of the molar Gag level). Cofilin binds to actin filaments and at increased pH levels has been shown to depolymerize actin microfilaments (29, 30, 51). The cofilin found inside virions could be complexed with actin filaments. It is interesting that the levels of cofilin roughly mirror the amount of β-actin in CEM_{ss}-produced virions, suggesting the possibility that cofilin is bound to cortical actin filaments in the CEM_{ss}-produced virions.

In addition to actin-binding proteins that regulate filament formation, our results found ezrin and moesin, two structural actin-binding proteins, inside virions. These proteins are members of the ezrin-radixin-moesin family of proteins that anchor cortical actin to the plasma membrane, thereby linking the cytoskeleton to the cell surface (1, 15, 23). In addition to full-length moesin, analysis of HIV-1 virions demonstrated that they contain a C-terminal fragment of moesin that appears to have been generated by the HIV-1 protease. The presence of this fragment suggests that moesin might also interact with the assembling and budding virion. The levels of full-length ezrin and moesin were similar for both types of virions (at approximately 2% of the molar Gag level) and were considerably lower than the actin levels. While most cell types express either ezrin or moesin, lymphocytes are unusual because they have been shown to express both molecules (4).

Several groups have shown that both ezrin and moesin are localized to microvilli on the surface of cells (4, 7, 15, 35). Microvilli are pseudopod-like structures that form on many types of activated cells and are regions of high membrane activity (44). A recent study has demonstrated that expression of moesin is required for microvillus formation (45). The presence of these microvillus-specific proteins inside HIV-1 virions suggests that virions may bud from these specialized structures. It has been recently shown that members of the ezrin-radixin-moesin protein family, as well as actin, are incorporated into rabies virus virions and that the rabies virus envelope protein colocalizes with these proteins in microvilli (40). Thus, budding from microvilli may be a general mechanism for several different enveloped viruses (9, 50). Other evidence for an association between HIV-1 budding and microvilli comes from a recent report of Pearce-Pratt et al. (36). Electron microscopy has observed HIV-1 budding from microvillus-like pseudopods on infected T cells that were activated by contact with epithelial cells (36). Both this report (36) and our results support the idea that HIV-1 virions bud from microvilli on lymphocytes.

The earliest observable stage of assembly starts with the coalescing of Gag precursors to form a flat sheet at the cortex of the plasma membrane (14) that ultimately buds from the cell surface. This is a complex and poorly understood process, which most probably requires interactions between Gag and cellular proteins that might result in the incorporation of host proteins into the virion. Early papers have postulated that cortical actin may play an active role in budding (9, 50); one hypothesis has actin forming the bud for the assembling virus (9). A recent study has shown that viral budding is diminished by cytochalasin D, an actin filament-depolymerizing compound (41). Combined with our data, these results suggest that the actin cytoskeleton is actively involved in efficient HIV-1 budding. The incorporation of cytoskeletal proteins into the virion appears to be specific since, by our analysis, other components of the cytoskeleton (e.g., thymosin β_4 [5, 8]) do not appear to be present inside the virions. Therefore, the assembling virion may interact with only a specific subset of the cytoskeletal proteins.

The presence of actin as well as regulatory and structural actin-binding proteins raises the possibility that the cytoskeleton is intact in the released virion and may play a role in infectivity. Experiments with cytoskeletal inhibitors are being carried out to examine this possibility.

The study of cytoskeletal proteins that are incorporated inside the HIV-1 virion has yielded some important leads toward the understanding of the assembly process. These results are being extended to determine if there is an active role for these proteins in assembly and budding.

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