Published December 7, 1998

Establishment of a Functional Human Immunodeficiency Virus Type 1 (HIV-1) Reverse Transcription Complex Involves the Cytoskeleton

By Alissa Bukrinskaya,*‡ Beda Brichacek,* Angela Mann,* and Mario Stevenson*

*From the *Program in Molecular Medicine, University of Massachusetts Medical Center, Worcester, Massachusetts 01605; and the ‡D.I. Ivanovsky Institute of Virology, Academy of Medical Science, Moscow 123098, Russia*

Summary

After interaction of human immunodeficiency virus type 1 (HIV-1) virions with cell surface receptors, a series of poorly characterized events results in establishment of a viral reverse transcription complex in the host cell cytoplasm. This process is coordinated in such a way that reverse transcription is initiated shortly after formation of the viral reverse transcription complex. However, the mechanism through which virus entry and initiation of reverse transcription are coordinated and how these events are compartmentalized in the infected cell are not known. In this study, we demonstrate that viral reverse transcription complexes associate rapidly with the host cell cytoskeleton during HIV-1 infection and that reverse transcription occurs almost entirely in the cytoskeletal compartment. Interruption of actin polymerization before virus infection reduced association of viral reverse transcription complexes with the cytoskeleton. In addition, efficient reverse transcription was dependent on intact actin microfilaments. The localization of reverse transcription to actin microfilaments was mediated by the interaction of a reverse transcription complex component (gag MA) with actin but not vimentin (intermediate filaments) or tubulin (microtubules). In addition, fusion, but not endocytosis-mediated HIV-1 infectivity, was impaired when actin depolymerizing agents were added to target cells before infection but not when added after infection. These results point to a previously unsuspected role for the host cell cytoskeleton in HIV-1 entry and suggest that components of the cytoskeleton promote establishment of the reverse transcription complex in the host cell and also the process of reverse transcription within this complex.

Key words: HIV-1 entry • reverse transcription • cytoskeleton

Efficient infection of a cell by the primate lentivirus HIV-1 involves receptor engagement by the virus and fusion between viral and host cell membranes (for a review, see reference 1). After this initial interaction, a poorly defined uncoating step leads to release of viral nucleic acids from the capsid (gag CA)[1] core. The uncoating process ultimately results in the formation of a reverse transcription complex that comprises the structural gag matrix (MA) protein (2–7) and the enzymatic proteins, reverse transcriptase and integrase (2, 3, 8), which are derived from the virion and in which viral cDNA synthesis proceeds (for a review, see reference 9). The structural gag MA protein is tightly associated with the reverse transcription complex, and conditions that promote dissociation of integrase do not affect association of gag MA (7). In addition, gag MA within reverse transcription complexes is myristoylated (10), and this modification is important in promoting membrane targeting of gag MA (within the context of gag polyprotein) to the plasma membrane (11–13). During virus entry, the membrane targeting property of myristoylated gag MA is regulated by phosphorylation (6, 10, 14). Nonphosphorylated gag MA is predominantly localized to the cell membrane. Phosphorylated gag MA, which is in viral reverse transcription complexes, is not membrane-associated (10). This likely allows free translocation of the reverse transcription complex from the point

[1]*Abbreviations used in this paper:* CA, capsid; CCA, CCD, and CCE, cytochalasin A, D, and E, respectively; FBS, fetal bovine serum; MA, matrix; MLV, murine leukemia virus; VSV, vesicular stomatitis virus.

A. Bukrinskaya and B. Brichacek contributed equally to this study.

Published December 7, 1998

of virus entry to the host cell nucleus (6, 10, 14). Gag MA has also been implicated in promoting the nuclear translocation of viral reverse transcription complexes. Mutations within a basic domain of gag MA that bears similarities to a canonical nuclear localization signal (2, 3) influence nuclear targeting of the reverse transcription complex in nondividing cells (2, 3, 5, 6, 15). However, mutations within the basic domain are not sufficient to prevent the nuclear translocation step (16, 17), suggesting that additional virion proteins promote translocation of the reverse transcription complex to the nucleus. In support of this, several groups have demonstrated that a virion-packaged accessory gene product, Vpr, also associates with the viral reverse transcription complex (5) and facilitates its nuclear translocation (5, 18–22). A cooperative model of MA/Vpr nuclear import has been suggested by the demonstration that gag MA binds to karyopherin in a Vpr-dependent manner (23). In addition, integrase has been shown to promote nuclear targeting of the reverse transcription complex in a cooperative manner with MA and Vpr proteins (18). While the actual roles played by gag MA in virus entry are still being defined, the protein represents a marker with which to monitor the fate of the viral reverse transcription complex during HIV-1 infection.

Several features of virus entry distinguish animal oncoretroviruses from primate lentiviruses. The virion core of oncoretroviruses and lentiviruses are comprised of gag CA protein. Although the reverse transcription complexes of oncoretroviruses such as murine leukemia virus (MLV) contain gag CA (24), those of HIV-1 contain gag MA (2–4, 6, 7). The process that effects this rearrangement of virion proteins during HIV-1 entry is not known. Similarly, there is little information on how the process of reverse transcription is initiated after formation of the reverse transcription complex within the target cell. Reverse transcription can be initiated within HIV-1 virions; however, the process is very inefficient and results predominantly in synthesis of early reverse transcripts (25–27). Although the processes of HIV-1 reverse transcription and nuclear translocation proceed concurrently, reverse transcription in vivo proceeds at ~1 nucleotide/s (28), whereas reverse transcription complexes translocate to the host cell nucleus as early as 1 h after infection (10, 29). Thus, after HIV-1 infection it is likely that the majority of reverse transcription complexes in the nucleus contain incomplete viral transcripts that are still undergoing reverse transcription. After MLV infection, where nuclear import is dependent on mitosis, the majority of reverse transcription is completed before nuclear import (30–32).

To better understand early events in the life cycle of HIV-1, we analyzed compartmentalization of viral reverse transcription complexes in the host cell early after virus infection. We demonstrate that viral reverse transcription complexes rapidly associate with actin microfilaments of the host cell cytoskeleton after infection. This association was necessary for efficient entry of viral nucleic acids into the target cell and for the efficient reverse transcription of genomic viral RNA. These observations suggest a model in which the cytoskeleton provides a cofactor or acts as a scaffold for the appropriate localization and activation of the viral reverse transcription complex and, ultimately, for efficient infection of the cell by HIV-1.

Materials and Methods

Generation of Viral Stocks. 293T cells (33) were maintained in DME supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), and 10% fetal bovine serum (FBS). For generation of viral stocks, 293T cells were transfected with HIV-1_{LAI} (34) proviral DNA (25 μg) using a modified calcium phosphate/DNA precipitation method (GIBCO BRL, Gaithersburg, MD) and incubated at 37°C. Virions in culture supernatants were collected at 24 and 48 h after transfection and either used directly for infection or amplified in MT-4 T cells in RPMI 1640 supplemented with 2 mM l-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% FBS. Pseudotype viruses were obtained after cotransfection of 293T cells with a vesicular stomatitis virus (VSV)-G envelope expression vector and an HIV-1_{LAI} clone containing a 600-bp deletion in gp120 coding sequences as described previously (35, 36). Calcium phosphate–transfected culture supernatants were collected at 48 h after transfection. Virus particles in culture supernatants were quantitated by measurement of reverse transcriptase activity. For preparation of ^{32}P-gag MA–labeled virions, productively infected $CD4^+$ MT-4 cells were incubated in the presence of 0.5 mCi ortho [^{32}P]phosphate per ml in phosphate-free medium containing 0.1% calf serum. After a 6-h incubation, culture supernatants were cleared of cellular debris by low-speed centrifugation (1,500 *g*, 10 min), and virions in clarified supernatants were harvested after high-speed centrifugation (10,000 *g*, 2 h).

Subcellular Fractionation. Subcellular fractions of HIV-1–infected MT-4 cells and HeLa-CD4-LTR/β-gal "Magi" cells comprising cell membrane, cytosolic, cytoskeletal, and nuclear extracts were prepared essentially as described elsewhere (20). Cells were pelleted and washed in a hypotonic solution (10 mM Hepes, pH 6.9, 10 mM KCl, 3 μl/ml aprotinin, 0.1 mM PMSF) and incubated on ice in hypotonic solution for 15–20 min. Cells were disrupted by dounce homogenization (20 strokes). Nuclei were pelleted at 1,000 *g* (3 min), supernatant was removed, and nuclei were further purified by three successive washes (retained as nuclear wash extract) in NTENT buffer (150 mM NaCl, 10 mM Tris-HCl, pH 7.2, 1 mM EDTA, 3 μl/ml aprotinin, 0.1 mM PMSF, 1% Triton X-100). Supernatant from pelleted nuclei was further centrifuged at 14,000 *g* for 30 min. The supernatant (cytosolic fraction) was separated, and the pellet was further resuspended in NTENT and recentrifuged at 14,000 *g* for 30 min. The resulting pellet comprises the cytoskeletal fraction, while the supernatant comprises the membrane fraction.

Western Blot Analysis. Subcellular fractions from an equivalent of 10^6 cells were adjusted to 62.5 mM Tris-HCl, pH 6.8, 0.2% SDS, 5% 2-ME, 10% glycerol. Samples were denatured by boiling and separated on 12% SDS-polyacrylamide gels. After electrophoresis, proteins were transferred to nitrocellulose (Hybond C Extra; Amersham Pharmacia Biotech, Inc., Piscataway, NY), blocked in 5% milk, and incubated with the respective antibody. Bound antibodies were visualized with peroxidase anti-Ig followed by enhanced chemiluminescence (ECL; Amersham Pharmacia Biotech, Inc.). All Western blot images were within the linear range of the ECL assay. Rhodamine-conjugated phalloidin and antibodies to tubulin, vimentin, actin, and myosin were obtained from Sigma Chemical Co. (St. Louis, MO). For

Published December 7, 1998

those experiments comparing protein distribution between different cellular compartments, cell lysate from an equivalent of 10^6 cells was resolved in each lane. This allowed for qualitative comparison of protein distribution between cellular compartments. For those experiments comparing the effect of cytoskeletal inhibitors on protein distribution within the same cellular compartment, the amount of total protein in each lane was adjusted to equivalent levels and, as a consequence, more quantitative comparisons could be made.

Analysis of In Vivo Interaction between Cytoskeletal and Virion Proteins. MT-4 cells were harvested 1.5 h after infection. Lysates of infected and uninfected cells were obtained by disruption of cells in immunoprecipitation buffer (50 mM Hepes, pH 6.9, 10 mM EDTA, 1% Triton X-100, 3 μl/ml aprotinin, 1 mM PMSF). The extracts were centrifuged at 1,000 *g* (3 min), and supernatants were precleared with normal rabbit antiserum (6 h, 4°C). Precleared lysates were adjusted to 100, 300, and 800 mM NaCl. Viral proteins were immunoprecipitated with rabbit anti-CA and anti-MA antibodies overnight at 4°C in the presence of protein A and G. Bound immunoprecipitates were washed five times in immunoprecipitation buffer and resolved on 15% SDS-PAGE gels. Proteins were transferred to Highbond C Extra (Amersham Pharmacia Biotech, Inc.) and analyzed by Western blotting with antibodies to tubulin, vimentin, actin, and myosin (Sigma Chemical Co.). Bound antibodies were visualized with peroxidase anti-Ig, followed by ECL (Amersham Pharmacia Biotech, Inc.).

Immunohistochemistry. Cells were seeded onto 12-well plates containing 0.13-mm-thick coverslips. Cells were grown for 24 h at 37°C in DME containing 10% FBS, Hygromycin (100 μg/ml), and G418 (200 μg/ml). For cytoskeletal staining, coverslips containing adherent cells were removed and rinsed two to three times with warm (37°C) PBS. Coverslips were placed in a petri dish and permeabilized with a solution of 0.1% Triton X-100 in PBS for 1 min, then washed twice with warm PBS. Cells were then immediately placed in warm (37°C), freshly prepared formaldehyde-Triton solution (4% formaldehyde, 0.2% Triton X-100, 60 mM Pipes, 25 mM Hepes, 10 mM EGTA, 3 mM $MgCl_2$, pH 6.1) for 10 min. The coverslips were then rinsed two to three times with room temperature PBS. To reduce nonspecific background staining, fixed cells were incubated with PBS containing 1% BSA for 15 min and washed twice with PBS before addition of staining solutions. For staining of actin microfilaments, cells were overlaid with 200 μl of a PBS/1% BSA solution containing 5 U/ml Texas Red phalloidin (Molecular Probes, Inc., Eugene, OR). Cells were incubated at room temperature for 20–30 min and then rinsed twice with PBS. For tubulin and vimentin visualization, permeabilized cells were overlaid with PBS/1% BSA containing tubulin (DM1A) and vimentin (V9) antibodies (Sigma Chemical Co.) for 20 min. Cells were washed twice in PBS and then incubated with FITC-conjugated donkey anti–mouse IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). Stained cells were mounted in Vectashield (Vector Laboratories, Inc., Burlingame, CA) to minimize photo-bleaching. Images were generated on a fluorescence microscope (Axioplan II; Carl Zeiss, Jena, Germany) using the appropriate FITC and Texas Red filter sets. Photographs were obtained with a Plan Apochromat objective (63× 1.40) under oil immersion.

PCR Analysis of HIV-1 cDNA Synthesis. Total cellular DNA was isolated from acutely infected cells using an IsoQuick DNA extraction protocol (Microprobe Corp., Bothell, WA). Minus-strand HIV-1 strong-stop cDNA products were amplified using LTR R (5′-g^{635} tgctagagattttccacactgac) and U5 (5′-g^{496} gctaactaggaacccactg) primers as described previously (37). Late reverse transcription products were amplified by LTR R (5′-g^{485} ggagctctctggctaact) and gag (5′-g^{931} gattaactgcgaatcgttc) primers. Viral cDNA was amplified in 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, and 0.001% (wt/vol) gelatin. After the initial denaturation (95°C, 2 min), amplification proceeded for 25 or 35 cycles of PCR (95°C, 30 s; 58°C, 30 s; 72°C, 1 min) followed by a final 5-min extension at 72°C. HIV-1 copy number standards were generated by PCR on serial twofold dilutions of 8E5 cells (each cell containing a defective provirus [38]). PCR products were Southern blotted onto nylon membranes and hybridized with a mixture of two ^{32}P-labeled oligonucleotide probes (5′-g^{566} tctgttgtgactctggt, 5′-g^{584} taactagagatccctcagac) for detection of both early and late products of reverse transcription. Primers are numbered according to the HIV-1_{HXB2} sequence (39). Hybridized PCR products were visualized on a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). Hybridized products were quantitated by volume integration (local mean background subtraction was applied) using ImageQuant software (Molecular Dynamics).

Single-cycle Infectivity Assays. HeLa-CD4-LTR/β-gal Magi indicator cells (35) were plated in 96-well microtiter plates (2×10^4 cells/well). HIV-1 virions (normalized to reverse transcription activity) were added in doubling dilutions to triplicate wells. At 48 h after infection, cells were harvested for quantitation of β-galactosidase production after hydrolysis of X-gal in vitro (Promega Corp., Madison, WI).

Results

Viral Reverse Transcription Complexes Rapidly Associate with the Host Cell Cytoskeleton. Gag MA is tightly associated with the viral transcription complex (2, 3, 7) likely through a high-affinity interaction with genomic viral RNA (40; our unpublished results), and thus represents a marker with which to follow the distribution of the reverse transcription complex in the infected cell. At 1 h after infection, MT-4 cells were fractionated into cytoplasmic, nuclear, cytoskeletal, and membrane fractions. The purity of the fractions was assessed by Western blotting with antibodies to cytoskeletal proteins (actin, tubulin), nuclear proteins (histone H1), and membrane proteins (CD4). Cytoskeletal proteins were detected primarily in cytosolic and cytoskeletal fractions and were absent from the nucleus (Fig. 1 *A*). Actin is present in the cell in two forms, as polymerized filaments and as unpolymerized monomers. Polymerized actin is enriched in cytoskeletal fractions, whereas much of the actin that is recovered from cytosolic extracts is in the unpolymerized form. The degree of actin polymerization varies between experiments and, as a consequence, the amount of actin recovered in cytosolic fractions varies between experiments. Histone H1 was present in nuclear fractions but not cytosolic or cytoskeletal fractions. CD4, the principal receptor for HIV-1, was restricted to membrane and cytoskeletal fractions, in agreement with previous reports (41, 42). Gag MA was found in all fractions with the exception of the cytoplasm (Fig. 1 *B*). We and others have previously demonstrated that gag MA that is associated with reverse transcription complexes is phosphorylated whereas that associated with membranes is not (6,

Published December 7, 1998

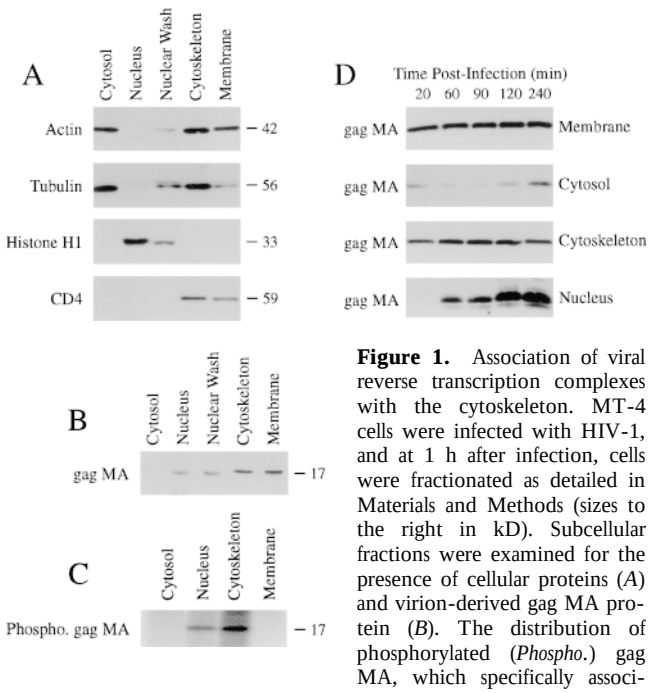

Figure 1. Association of viral reverse transcription complexes with the cytoskeleton. MT-4 cells were infected with HIV-1, and at 1 h after infection, cells were fractionated as detailed in Materials and Methods (sizes to the right in kD). Subcellular fractions were examined for the presence of cellular proteins (*A*) and virion-derived gag MA protein (*B*). The distribution of phosphorylated (*Phospho.*) gag MA, which specifically associates with the reverse transcription complex, is shown in *C*. MT-4 cells were fractionated at various intervals after HIV-1 infection, and the distribution of gag MA in various cellular compartments was determined by Western blotting (*D*). All Western blot images are within the linear range of the ECL assay. For *A*, *B*, and *C*, cell lysate from a total of 10^6 cells was loaded in each lane.

10, 14). Upon infection of cells with HIV-1 virions containing ^{32}P-gag MA, phosphorylated gag MA protein was localized exclusively to the nucleus and to the cytoskeleton (Fig. 1 *C*). In agreement with our previously published results (10), phosphorylated gag MA was excluded from the membrane despite the fact that the majority of gag MA in the acutely infected cell remains membrane associated (Fig. 1, *B* and *C*). Thus, gag MA in the cytoskeletal fraction was associated with viral reverse transcription complexes and did not represent contamination of that fraction with membrane-associated virion proteins. To examine the kinetics with which viral reverse transcription complexes associated with the cytoskeleton and the nucleus, cells were infected and subcellular fractions were prepared at early intervals after infection. Gag MA appeared in the cytoskeleton within 20 min of infection (Fig. 1 *D*), whereas its appearance in the nucleus was not evident until 60 min after infection (Fig. 1 *D*).

We next determined whether the association of the reverse transcription complex with the cytoskeleton was sensitive to cytoskeleton-disrupting agents. MT-4 cells were treated for 2 h with cytochalasin D (CCD, 5 μM), a specific inhibitor of actin polymerization (43, 44). Cells were then infected with HIV-1 and infection was allowed to proceed in the absence of CCD. At 1 h after infection, there was a reduction in the amount of gag MA present in the cytoskeletal and nuclear fractions, whereas there was a slight increase in the amount of gag MA associated with the membrane (Fig. 2 *A*). Since infection proceeded in the absence of CCD and the effects of CCD are reversible, there was a slight restoration of gag MA association with the cytoskeleton and nucleus when the distribution was examined at 5 h after infection (Fig. 2 *A*). In comparison, treatment of cells with demecolcine, which causes perinuclear aggregation of intermediate filaments (45), or with nocodazole, a specific inhibitor of tubulin polymerization into microtubules (46), did not significantly affect the association of gag MA with the cytoskeleton (Fig. 2 *B*). The inhibitory effects of CCD were dependent on the time of drug addition. When cells were treated with CCD for either 1 or 2 h before virus infection, the amount of gag MA in the cytoskeleton and in the nucleus was markedly reduced (Fig. 2 *C*). The presence of gag MA in the cytoskeleton or nucleus was not significantly affected if CCD was added as early as 1 h after infection (Fig. 2 *C*). Treatment of cells with CCD inhibits actin polymerization; however, monomeric actin could still be recovered from the cytoskeletal fraction of CCD-treated cells (Fig. 2 *D*). In addition, inhibition of actin polymerization resulted in an increase in the amount of actin recovered from the cytosolic fraction, likely as a consequence of increased levels of monomeric actin. The presence of gag MA in the cytoskeletal and nuclear fractions of cells treated with CCD after virus infection suggests that the interaction between the reverse transcription complex and the cytoskeleton is retained whether actin is monomeric or polymerized. Since gag MA rapidly associates with the cytoskeleton, and CCD did not affect this association when added 1 h after infection, the results suggest that, once established, the interaction between the reverse transcription complex and the cytoskeleton is not reversed upon actin depolymerization.

Interaction between Gag MA and Actin In Vivo. We next investigated the mechanism by which viral reverse transcription complexes associated with the cytoskeleton. Recent studies have suggested an interaction between HIV-1 gag polyprotein and F-actin present in microfilaments (47). Therefore, we determined whether the two major processed products of gag polyprotein in the virion, namely gag MA and gag CA, associated with actin in vivo. Cell extracts were prepared from uninfected MT-4 cells and from MT-4 cells 1 h after HIV-1 infection. Lysates of infected and uninfected cells were adjusted to 100, 300, and 800 mM NaCl and immunoprecipitated with antibodies to gag MA or gag CA (Fig. 3 *A*). The presence of cytoskeletal proteins in these immunoprecipitates was then determined by Western blotting with antibodies to cytoskeletal proteins including actin (microfilaments), vimentin (intermediate filaments), or tubulin (microtubules) and to gag MA and gag CA as outlined in Fig. 3 *A*. Antibodies to actin reacted with gag MA immunoprecipitates from HIV-1–infected cells at 100, 300, and 800 mM NaCl, suggesting a tight interaction between actin and gag MA (Fig. 3 *B*). Gag MA immunoprecipitates from uninfected cells also reacted with actin antibody but only at 100 mM NaCl, likely a result of a nonspecific interaction between actin and a cellular protein within gag MA immunoprecipitates (Fig. 3 *B*). In comparison, antibodies to actin bound to gag CA immuno-

Published December 7, 1998

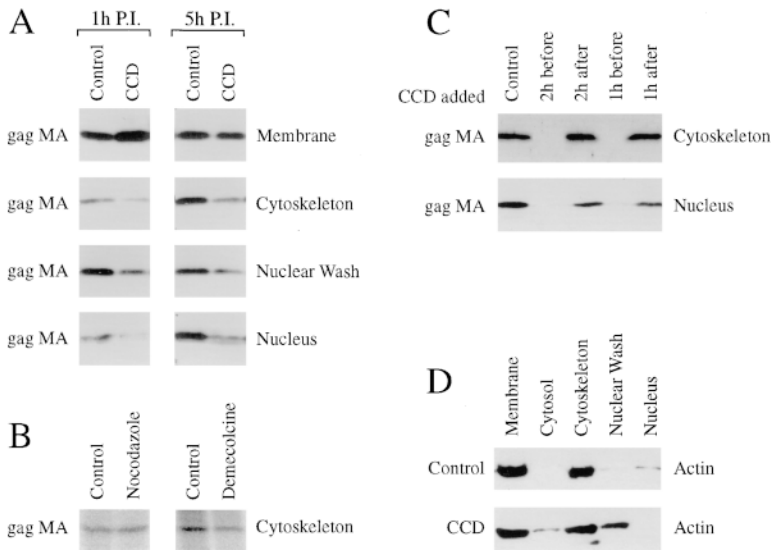

Figure 2. Inhibitors of actin polymerization reduce cytoskeletal association of viral reverse transcription complexes. (*A*) MT-4 cells were treated for 2 h with CCD (5 μM), which prevents actin polymerization. Cells were then infected with HIV-1 and fractionated at 1 and 5 h after infection (*P.I.*) for analysis of gag MA distribution. (*B*) MT-4 cells were treated with nocodazole (10 μM), an inhibitor of tubulin polymerization into microtubules, or demecolcine (10 μM), which causes disaggregation of intermediate filaments. Gag MA in the cytoskeleton was examined by Western blotting 1 h after infection. (*C*) MT-4 cells were incubated with CCD for 1 and 2 h before infection or 1 and 2 h after infection. At 3 h after infection, cytoskeletal and nuclear extracts were prepared and examined for the presence of gag MA by Western blotting. (*D*) The subcellular distribution of actin was examined by Western blotting in MT-4 cells (*Control*) and in MT-4 cells after a 2-h incubation with CCD. For *A*, *B*, and *C*, equal amounts of cellular protein were loaded in each lane. In *D*, each lane contains lysate from 10^6 cells.

precipitates prepared in 100 mM NaCl but not in 300 or 800 mM NaCl. This weak interaction was again nonspecific since it was evident in both infected and uninfected cells (Fig. 3 *B*). No reactivity of gag MA or gag CA immunoprecipitates was noted with antibodies to vimentin or to tubulin (Fig. 3 *B*), suggesting that there was no association between these proteins in acutely infected cells. Each antibody recognized its cognate protein at all NaCl concentrations, indicating that the high salt immunoprecipitation conditions did not compromise antibody reactivity (Fig. 3 *C*). Collectively, these results suggest that gag MA associates with actin during early stages of HIV-1 infection.

Efficient Reverse Transcription of HIV-1 Nucleic Acids Involves the Cytoskeleton. To determine whether the cytoskeletal association of viral reverse transcription complexes influenced the function of this complex, we initially determined whether cytoskeletal depolymerizing agents influenced cDNA synthesis within the complex. According to current models of reverse transcription (48), the earliest viral cDNA detectable in the cell after fusion and uncoating is minus-strand strong-stop cDNA which spans the R-U5 region of the viral LTR (Fig. 4 *A*), whereas a late step in reverse transcription involves synthesis of plus-strand DNA spanning the LTR U5/gag boundary (Fig. 4 *A*). Thus, LTR R-U5 products represent a marker with which to gauge the extent of virus entry and early reverse transcription, whereas LTR U5-gag products provide an indicator of the extent of late reverse transcription in the infected cell (37). CCD (5 μM) was added to cells 1 h before or 2 h after virus infection. Cells were harvested 4 h after virus infection for isolation of viral nucleic acids from membrane/cytosol, cytoskeletal, and nuclear extracts. Early (LTR R-U5)

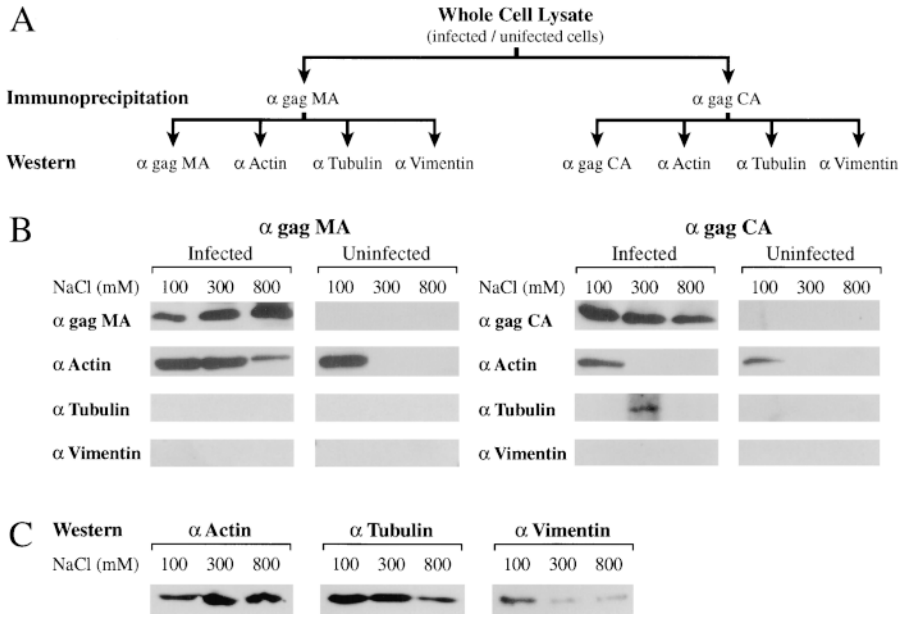

Figure 3. Interaction between cytoskeletal proteins and HIV-1 gag MA in vivo. (*A*) Cell lysates of HIV-1–infected and uninfected MT-4 cells were immunoprecipitated with antibodies to gag MA and gag CA in 100, 300, and 800 mM NaCl. The presence of viral and cytoskeletal proteins in gag MA and gag CA immune complexes was examined by Western blotting (*B*) with antibodies to viral (gag MA, gag CA) and cytoskeletal (actin, tubulin, vimentin) proteins. (*C*) Western blot analysis of cytoskeletal proteins in cell lysates at different NaCl concentrations.

Published December 7, 1998

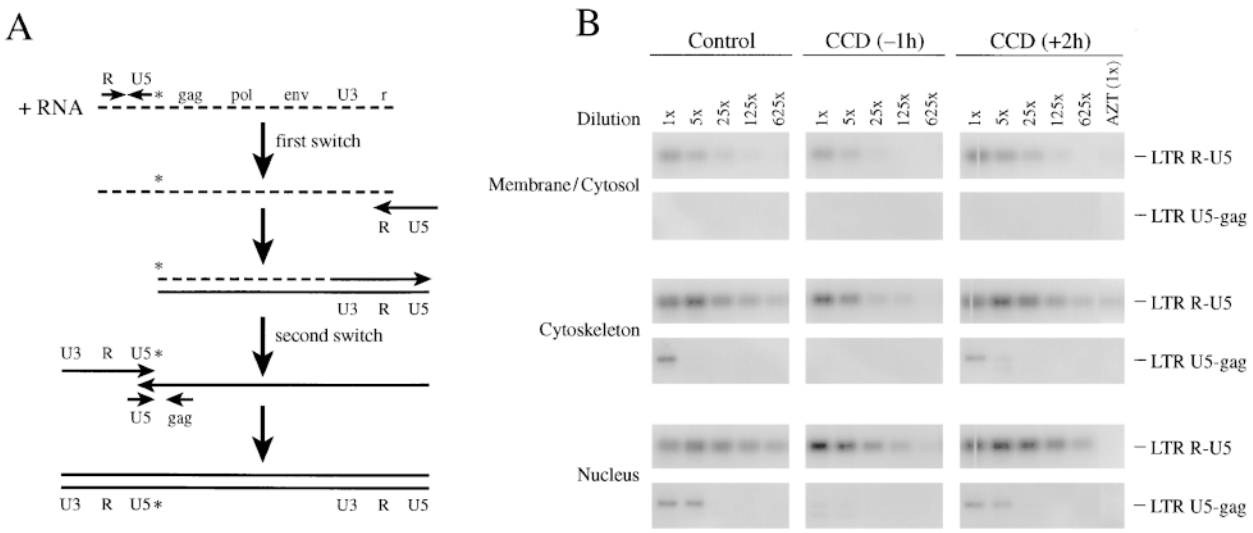

Figure 4. Efficient HIV-1 reverse transcription requires actin microfilaments. (*A*) Schematic of the major steps involved in synthesis of HIV-1 cDNA. *Broken lines*, genomic RNA; *solid lines*, cDNA; *short arrows*, regions of the viral genome to which PCR primers are directed; *asterisk*, the primer binding site. R-U5 and U5-gag primers amplify predominantly early and late products of reverse transcription, respectively. (*B*) HIV-1–infected MT-4 cells were incubated in the presence or absence of CCD (5 μM, added 1 h before or 2 h after HIV-1 infection), and subcellular fractions were prepared at 3 h after infection. Lysates were treated with DNAse to remove carryover DNA in the inoculum (reference 37). Early (LTR R-U5) and late (LTR U5-gag) products of reverse transcription in cell fractions were quantitated by PCR in serial fivefold dilutions of the cellular lysate. Infections carried out in the presence of AZT (5 μM) were used to verify de novo synthesis of viral cDNA. PCR products were visualized by Southern blot hybridization to HIV-1–specific oligonucleotide probes as outlined in Materials and Methods.

and late (LTR U5-gag) cDNA products of reverse transcription in these extracts were analyzed by PCR under limiting dilution conditions. In untreated (control) cells, early reverse transcripts were detectable in all subcellular fractions (Fig. 4 *B*). Surprisingly, late products of reverse transcription were found only in cytoskeletal and nuclear fractions but were absent from membrane/cytosol extracts (Fig. 4 *B*). When added 1 h before infection, CCD reduced the amount of early reverse transcripts in all fractions by approximately four- to fivefold (Fig. 4 *B*). Late products were not detectable in the cytoskeleton of CCD-treated cultures, whereas in the nucleus their abundance was reduced by ~25-fold (Fig. 4 *B*). In contrast, addition of CCD to cells 2 h after virus infection had no effect on abundance of either early or late products of reverse transcription in any cell fraction (Fig. 4 *B*). Both early and late cDNA products were the result of de novo reverse transcription rather than virion-associated cDNA products, since the amount of PCR products was markedly reduced in cultures that had been pretreated with the reverse transcriptase inhibitor, AZT (Fig. 4 *B*). The results suggest that steps in virus entry leading up to initiation of reverse transcription (fusion/uncoating, activation of the reverse transcription complex) involve the cytoskeleton. The results further indicate that the reverse transcription process is localized primarily on the cytoskeleton and that efficient synthesis of late cDNA transcripts requires the presence of actin microfilaments. This interaction with the cytoskeleton appears to be initiated early upon infection and is also irreversible, since addition of CCD to cells after virus infection did not compromise the reverse transcription process (Fig. 4 *B*).

Efficient HIV-1 Infection Requires an Intact Cytoskeleton. A confounding factor in experiments that examine early events in the virus life cycle is that much of the viral material present in the inoculum is noninfectious. Thus, it is possible that the viral nucleic acids and proteins found in association with the cytoskeleton may not be the component of the inoculum that was actually mediating bona fide virus infection. To address this issue, we examined the effects of cytoskeletal inhibitors on HIV-1 infection. After completion of reverse transcription, viral nucleic acids integrate into host cell DNA, leading to the formation of a "provirus" from which genomic and subgenomic viral transcripts are derived. Therefore, we examined the effects of cytoskeletal inhibitors on provirus establishment in a single-cycle Magi infectivity assay (35). We initially determined drug concentrations required to impair organization of their cognate cytoskeletal targets in Magi cells (Fig. 5 *A*). In adherent cells, actin is organized in "stress fibers" of polymerized actin microfilaments (Fig. 5 *A*). Treatment of cells with cytochalasins A, D, and E (5 μM) led to a collapse of actin microfilaments into condensed bundles of monomeric actin that was randomly distributed through the cell cytoplasm (Fig. 5 *A*). This concentration of cytochalasin did not compromise cell viability during the course of the experiment (not shown). Complete disruption of actin microfilaments typically occurred within 1 h of drug addition. All three cytochalasins (CCA, CCD, and CCE) markedly impaired viral infectivity when added 2 h before (*2h pre*) virus infection (Fig. 5 *B*). In contrast, the drugs did not significantly affect HIV-1 infectivity when added 2 h after (*2h post*) virus infection, and in some cases enhanced

Published December 7, 1998

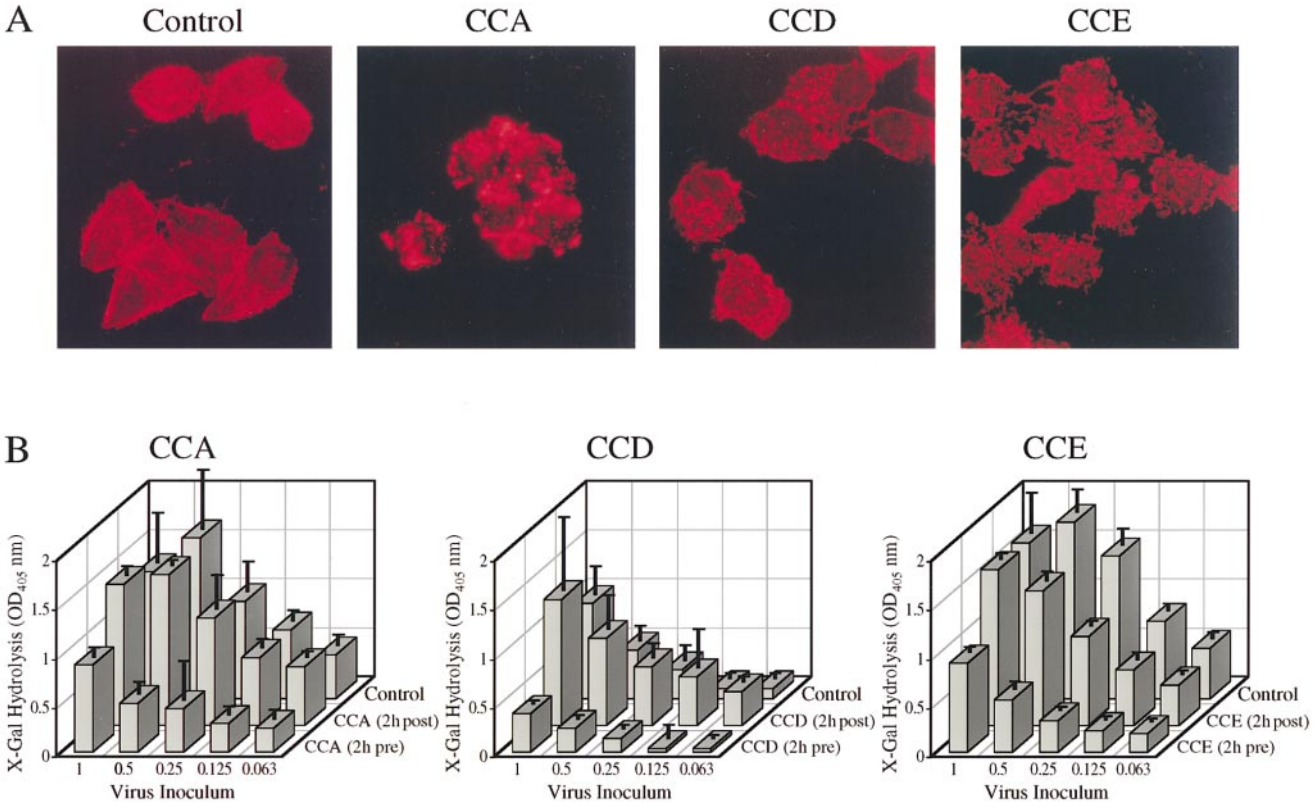

Figure 5. Actin microfilaments are required for efficient HIV-1 infection. (*A*) Immunohistochemical analysis of HeLa-CD4-LTR/β-gal indicator cells with rhodamine-phalloidin after a 1-h incubation with the actin depolymerizing agents CCA, CCD, and CCE (5 μM). In untreated (*Control*) cells, actin filaments are organized in linear array stress fibers of polymerized actin. Cytochalasin (5 μM) was sufficient to almost completely prevent actin polymerization in these cells. (*B*) Magi cells were incubated with the indicated cytochalasins for 2 h before (*2h pre*) and 2 h after (*2h post*) virus infection. Infected cells were harvested 48 h later for quantitation of β-galactosidase production. Cell infection was evaluated at five different levels of input virus in triplicate wells.

infection (Fig. 5 *B*). Thus, the inhibitory effects of the cytochalasins on HIV-1 infectivity were not due, for example, to indirect effects of the drugs on host cell viability. This pattern of inhibition is in strong agreement with the effect of these inhibitors on the association of gag MA with the cytoskeleton (Figs. 2 and 3) and on reverse transcription (Fig. 4) in MT-4 cells, in that CCD was inhibitory when added at least 1 h before but not after virus infection. We next determined whether there was a concomitant effect on the reverse transcription process in Magi cells. Early (LTR R-U5) and late (LTR U5-gag) products of reverse transcription were quantitated at different intervals after infection (Fig. 6). Treatment of CCD only slightly impaired the levels of minus-strand strong-stop cDNA in all fractions (Fig. 6). In agreement with the pattern observed in MT-4 cells, late products of reverse transcription were evident only in the cytoskeleton and the nucleus. However, pretreatment of the cells with CCD delayed the appearance of late products of reverse transcription (Fig. 6). In untreated cells, late products of reverse transcription were detectable in the cytoskeleton and the nucleus at 1 and 3 h after infection, respectively. After CCD treatment, late reverse transcription products could not be detected until 5 h after infection (Fig. 6). In contrast to the inhibitory effects of cytochalasins on HIV-1 entry, the microtubule disrupting agent, nocodazole, had a very modest inhibitory effect on HIV-1 infectivity in several independent experiments (Fig. 7). Although a slight inhibitory effect on HIV-1 infectivity was observed at lower levels of virus inoculum, these effects were equally apparent whether nocodazole was added before or after HIV-1 infection. Since nocodazole had no effect on the interaction between gag MA and the cytoskeleton or on the abundance of gag MA in the acutely infected cell (Fig. 2 *B*), it is unlikely that nocodazole is directly influencing establishment of the reverse transcription complex. Since the slight inhibitory effect was evident when the inhibitor was added before and after virus infection, we cannot exclude the possibility that nocodazole influenced the infectivity assay indirectly through its effect on host cell function. Collectively, these results indicate that the reverse transcription process in a newly infected cell is localized to the host cell cytoskeleton and that intact actin microfilaments are necessary for efficient reverse transcription.

Actin–Myosin Interaction Influences HIV-1 Infection. Translational movement along actin microfilaments is mediated by myosins (for a review, see reference 49). Myosin function is regulated by phosphorylation of the regulatory light

Published December 7, 1998

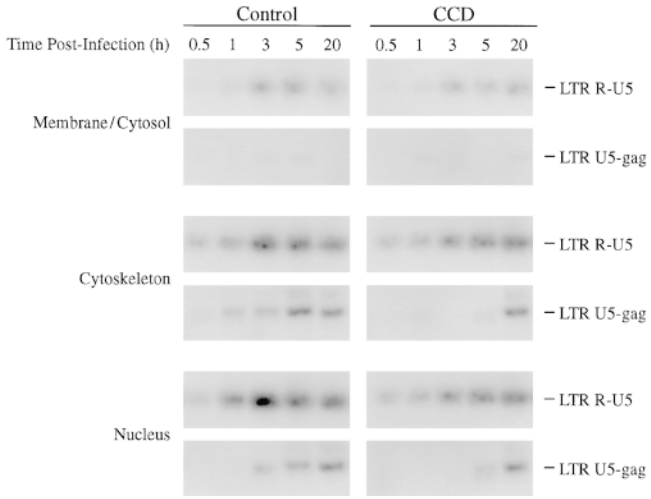

Figure 6. Efficient synthesis of late reverse transcription products requires actin microfilaments. Untreated (*Control*) and CCD-treated Magi cells (5 μM CCD added 2 h before virus infection) were harvested at different intervals after infection, and early and late products of reverse transcription in different subcellular compartments were examined by PCR using LTR R-U5 and LTR U5-gag primers, respectively.

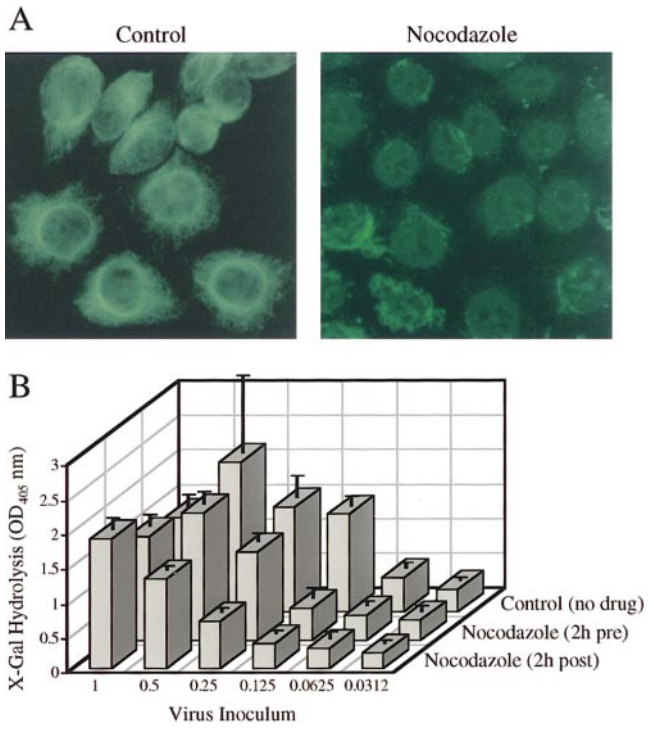

Figure 7. HIV-1 infection does not require microtubules. (*A*) Organization of microtubules in Magi cells after incubation (2 h) with nocodazole, a microtubule depolymerizing drug that fixes monomers of tubulin. (*B*) Single-cycle HIV-1 infectivity characteristics in untreated and nocodazole-treated Magi cells. Infectivity was analyzed in triplicate at six different levels of input virus. Effects of nocodazole on single-cycle HIV-1 infection of Magi cells are as outlined in the legend to Fig. 5.

chains by myosin light-chain kinase (50), which promotes myosin ATPase activity and polymerization of actin cables (51, 52). ML7 (53) and KT5926 (54; both obtained from Calbiochem Corp., La Jolla, CA) are well-described inhibitors of myosin light-chain kinase (K_i; 21 μM and 18 nM, respectively). Inhibition of myosin light-chain phosphorylation inhibits myosin-mediated translational movement. Thus, we examined whether HIV-1 infectivity was sensitive to agents that affect myosin-mediated translational movement along actin microfilaments. The myosin light-chain kinase inhibitors ML7 (Fig. 8 *A*) and KT5926 (Fig. 8 *B*) both impaired viral infectivity. This inhibition was similar to that observed with cytochalasins, in that ML7 inhibited HIV-1 infection when added 2 h before virus infection, but not 2 h after infection. Subcellular distribution of viral reverse transcription complexes in cells treated with KT5926 was determined in order to identify at which point the inhibition of infectivity was manifest. Pretreatment of cells with increasing concentrations of KT5926 between 100 and 500 nM resulted in a gradual decrease in the amount of gag MA that localized to the host cell nucleus, while there was a corresponding increase in the level of gag MA in the cytoskeletal fraction (Fig. 8 *C*). One explanation for this result is that inhibition of myosin phosphorylation delays the transit of viral reverse transcription complexes from the cytoskeleton to the nucleus. KT5926 also reduced the amount of gag MA that was present in the cytosol and membrane fractions by ~30%, indicating that viral uptake was also being influenced (Fig. 8 *C*). However, this modest effect on gag MA uptake was not dose dependent and was unlikely to account for the rather dramatic effect of this inhibitor on virus infection. Thus, although additional effects of the inhibitor on virus uptake cannot be excluded, the inhibitory profiles are more consistent with the hypothesis that the effects of the inhibitor on gag MA redistribution between cytoskeletal and nuclear fractions account for the majority of the inhibitory effects on virus infection.

The Host Cell Cytoskeleton Is Important for HIV-1 Entry by Fusion but not by Endocytosis. HIV-1 entry is mediated by pH-independent fusion between viral and host cell membranes (55). When HIV-1 virion cores are pseudotyped with envelope proteins of VSV, infection is mediated by endocytosis (35, 56). Thus, we compared the relative cytoskeletal requirements for virus entry via the fusion and endocytosis routes. In contrast to the marked effects of CCD on wild-type HIV-1 infection (Fig. 5 *B*), CCD had no significant effect on entry of VSV-pseudotyped HIV-1 when added before virus inoculation (Fig. 9 *A*). Similarly, CCD had no effect on the association of gag MA with the cytoskeleton (Fig. 9 *B*). Although a slight inhibitory effect on virus infection was evident in cultures in which the inhibitor was added 2 h after virus inoculation (Fig. 9 *A*), this very slight effect was not consistent over several independent infectivity experiments and likely fell within the variability of the single-cycle infectivity assay. These results suggest that cytoskeletal association can be uncoupled from infectivity when HIV-1 entry is mediated by endocytosis. It is possible that engagement of the cytoskeleton is coupled to fusion, which places the reverse transcription com-

Published December 7, 1998

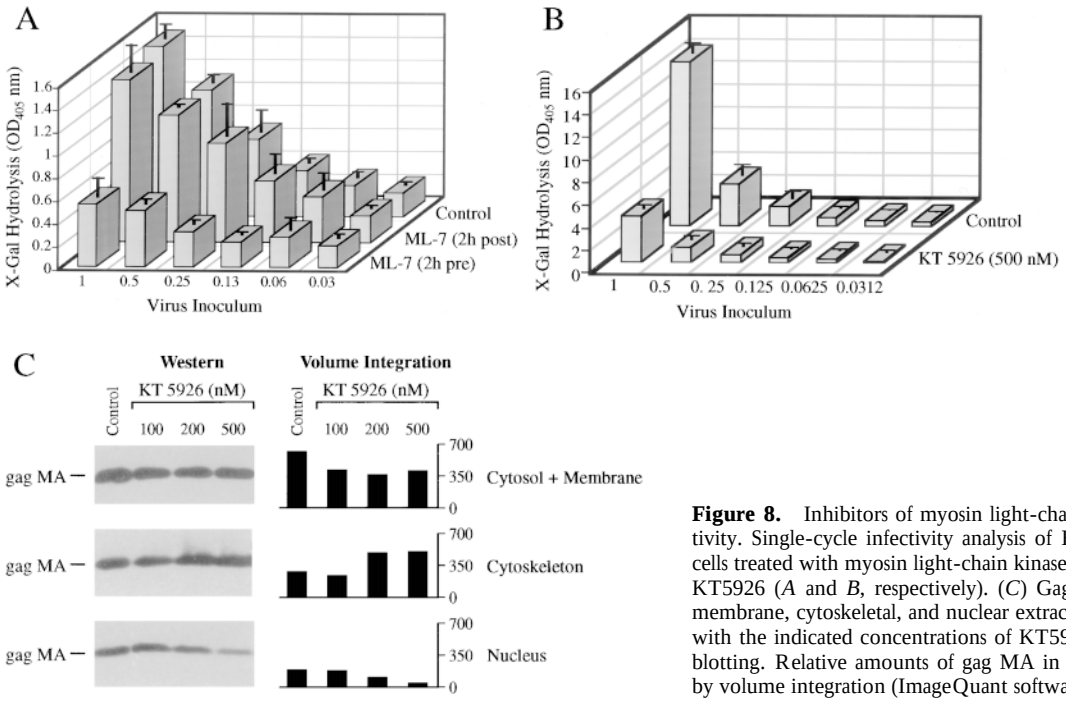

Figure 8. Inhibitors of myosin light-chain kinase impair HIV-1 infectivity. Single-cycle infectivity analysis of HIV-1 was examined in Magi cells treated with myosin light-chain kinase inhibitors ML-7 (20 μM) and KT5926 (*A* and *B*, respectively). (*C*) Gag MA distribution in cytosol/membrane, cytoskeletal, and nuclear extracts of Magi cells after treatment with the indicated concentrations of KT5926 was examined by Western blotting. Relative amounts of gag MA in each sample were determined by volume integration (ImageQuant software; Molecular Dynamics).

plex in an appropriate context within the acutely infected cell (i.e., in association with the cytoskeleton). The experiments also indicate that, upon establishment of a functional reverse transcription complex within the cell (whether after fusion or endocytosis), events in the viral life cycle leading up to de novo gene expression from the integrated provirus do not require actin microfilaments.

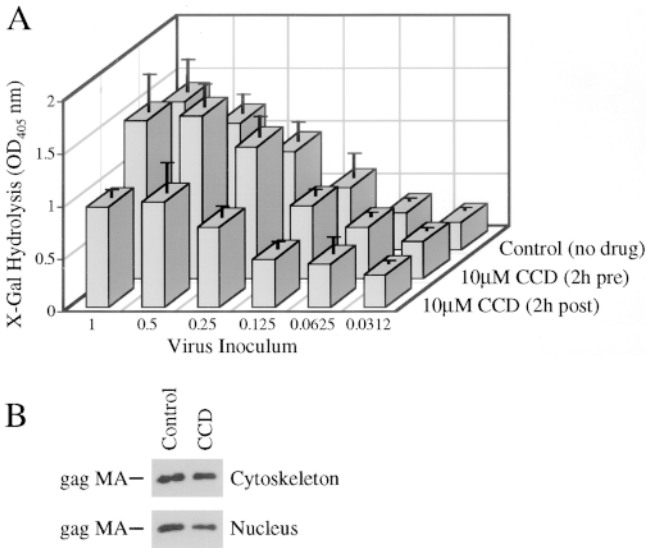

Figure 9. Actin microfilaments are not required for HIV-1 infection via endocytosis. (*A*) Effect of CCD on single-cycle infectivity of VSV-G–pseudotyped HIV-1. Virions containing VSV envelope proteins were obtained after cotransfection of an HIV-1 envelope minus mutant with VSV-G expression vector. Pseudotyped virions were obtained from transfected cell supernatants, and infectivities were determined in CCD-treated and untreated Magi cells. (*B*) Untreated (*Control*) and CCD-treated (10 μM CCD added 2 h before infection) Magi cells were infected with VSV-G–pseudotyped HIV-1 virions. Cytoskeletal and nuclear extracts were prepared 4 h after infection for analysis of gag MA distribution by Western blotting.

Discussion

The results outlined in this study suggest that an essential early event in the virus life cycle, namely establishment of the viral reverse transcription complex, is promoted by actin microfilaments of the host cell cytoskeleton. During preparation of this manuscript, Iyengar et al. (57) demonstrated that HIV-1 infection of primary T cells could be completely inhibited by treatment of cells with CCD. The investigators went on to demonstrate that colocalization of the HIV-1 receptors, CD4 and CXCR4, on primary T cells required actin, and suggested that actin is necessary for a step leading up to fusion between viral and host cell membranes. However, the results presented here clearly indicate that the inhibitory effects of the cytochalasins in T cells and Magi cells can only be partly accounted for by inhibition of a step leading up to entry of the reverse transcription complex. In the experiments presented here, minus-strand strong-stop cDNA was detected in CCD-treated T cells and Magi cells which completely lack actin microfilaments. Our results indicate that the reverse transcription complex rapidly associates with actin microfilaments and that efficient reverse transcription is dependent on the presence of actin microfilaments. Our results also suggest that actin microfilaments of the host cell cytoskeleton are a major site for reverse transcription in the infected cell. Although actin depolymerization did not significantly affect the presence of early reverse transcription products in the cytoskeleton and in the nucleus, these early reverse

Published December 7, 1998

transcription products did not go on to complete reverse transcription if actin polymerization was inhibited before virus infection. This suggests that interaction with actin microfilaments is an essential intermediate step that is required for efficient reverse transcription, and early reverse transcripts that bypass this intermediate step still translocate to the nucleus but are incapable of undergoing complete reverse transcription. The basis for the dependence of an intact cytoskeleton for efficient reverse transcription is unclear at present, and we know of no precedent in the animal oncoretrovirus or primate lentivirus systems to explain this dependence. Cytoskeletal proteins have also been implicated in the activation of transcription of paramyxoviruses (measles virus, Sendai virus, respiratory syncytial virus) and rhabdoviruses (VSV) (58–60). For example, in paramyxoviruses such as human parainfluenza virus type 3, polymerized actin has been shown to be necessary for the activation of transcription from the ribonucleoprotein complex in vitro and in vivo (61–63). It has been suggested that upon interaction with actin, the paramyxovirus ribonucleoprotein complex undergoes a conformational alteration from a loosely coiled to a moderately condensed form, which facilitates transcription (62). It is possible that activation of HIV-1 reverse transcription also requires a conformational change in the reverse transcription complex, and that this is mediated through interaction with the actin microfilaments. Although there is no direct evidence to suggest that activation of the reverse transcription complex requires interaction with the cytoskeleton, such a model is suggested by the demonstration that the large subunit of HIV-1 reverse transcriptase interacts with β-actin (64). Although this interaction has been implicated in promoting the release of virus particles from the infected cell, in light of our current findings it is tempting to speculate that such an interaction may provide the basis by which association with actin microfilaments was required for efficient reverse transcription. Alternatively, the uncoating process itself may be facilitated by association of the incoming capsid core with the cytoskeleton, due to the interaction between capsid protein and actin. However, the results presented here would favor the former hypothesis in that gag MA within the reverse transcription complex associated with actin, whereas capsid, the major component of the viral core, did not exhibit any significant binding. The infectious virus particle does not contain all the components necessary for efficient completion of cDNA synthesis, since only early reverse transcription products are reconstituted in in vitro reverse transcription reactions using extracts from disrupted virions (for a review, see reference 65). Thus, it is also possible that the cytoskeleton is a favored site for reverse transcription because cellular factors important in the reverse transcription process may also localize to the cytoskeleton.

VSV-G envelope–pseudotyped HIV-1 entry was independent of actin microfilaments, suggesting that virus entry by endocytosis circumvents the aspect of the virus life cycle that requires cytoskeletal interaction. Entry of pseudotyped HIV-1 by endocytosis requires an acidification step which promotes fusion between the viral membrane and the membrane of the endocytic vesicle (56). It is possible that the low pH environment of the endosome may also alter conformation of the viral reverse transcription complex in a way that obviates the requirement for actin in this process. By analogy, the requirement for actin microfilaments in the entry of MLV also depends on the route of virus entry. MLV infection of NIH 3T3 cells occurs by endocytosis, whereas that of XC Sarcoma cells proceeds by surface fusion. Both entry mechanisms are dependent on polymerized actin, implicating a critical role for this network in an early step common to both entry pathways, perhaps at the level of receptor clustering (66, 67). However, events in the entry process subsequent to virus uptake via endocytosis, but not by fusion, were dependent on intact microtubules, implicating the microtubule component of the cytoskeleton in a postpenetration step (67).

Previous studies have implicated the host cell cytoskeleton in late events in the virus life cycle. The release of HIV-1 from polarized cells was shown to be dependent on an intact cytoskeleton (68), whereas inhibitors of myosin light-chain kinase and actin polymerization inhibited the release of HIV-1 from the infected cells (69). Polymerized actin has been implicated in directional budding of HIV-1 from infected monocytes as evidenced by colocalization of actin and gag CA protein at the sites of virus release (23). A role for the cytoskeleton in virus release was also suggested by the observation that HIV-1 gag polyprotein associated with polymerized actin (47) and that HIV-1 virions contain actin in a 10% molar ratio relative to gag polyproteins (70). The accessory gene product, Vif, which has an important role in the regulation of virus infectivity, has been proposed to regulate virus maturation by mediating interaction with the cellular cytoskeleton (71). The role of gag–cytoskeleton interaction in virus maturation may be paradoxical with the potential role of gag MA–cytoskeletal interaction during virus entry. Presumably, the interaction between gag precursor proteins and the cytoskeleton at late stages in the virus life cycle would favor accumulation of virion precursor proteins at the cell surface, which is necessary for efficient budding from the infected cell. However, as shown in this study, at early stages in the virus life cycle this interaction appears to be promoting the establishment of infection after virus uptake. Our data suggest that phosphorylated gag MA proteins of the virion associate with the cytoskeleton. This differential phosphorylation likely dictates the fate of the replication intermediates that have associated with the cytoskeleton. We have previously suggested a model in which gag MA phosphorylation regulates the opposing targeting properties of this protein during virus assembly and during virus entry (10, 14).

Recent studies have suggested that nuclear targeting of incoming human foamy virus proteins was dependent on the presence of intact microtubules (72). Similar to the localization of viral reverse transcription complexes to actin microfilaments outlined here, the study of Saib et al. (72) demonstrated aggregation of viral reverse transcription complexes on microtubules. That study also suggested that virus

entry was not dependent on microtubules but that a subsequent step, either at the level of reverse transcription or nuclear import, was microtubule dependent and highly sensitive to colchicine, a microtubule-disrupting agent. These results are reminiscent of observations in other viral systems such as herpes simplex, where transport of incoming viral capsids to the nucleus is mediated by microtubules (73). Intriguingly, recent studies in our laboratory (our unpublished results) suggest that intermediate filaments, another component of the cytoskeleton, do not appear to be involved in events leading up to completion of viral cDNA synthesis on the cytoskeleton but may be important for subsequent translocation of nascent viral reverse transcription complexes to the host cell nucleus.

We thank M. Emerman for the pSfi clone and the VSV-G expression vector, S. Doxsey for guidance in the cytoskeletal analysis, and L. Henderson for the rabbit anti–HIV-1 gag MA antisera. Magi cells, 293T cells, and MT-4 T cells were provided by the National Institutes of Health AIDS Research and Reference Reagent Program (National Institute of Allergy and Infectious Diseases, Bethesda, MD).

This work was supported by grants from the National Institutes of Health and the Fogarty Program to M. Stevenson.

Address correspondence to Mario Stevenson, Department of Molecular Genetics and Microbiology, Program in Molecular Medicine, University of Massachusetts Medical Center, 373 Plantation St., Worcester, MA 01605. Phone: 508-856-4582; Fax: 508-856-4075.

Received for publication 20 July 1998 and in revised form 9 September 1998.

References

1. Dragic, T., A. Trkola, and J.P. Moore. 1997. HIV co-receptors: gateways to the cell. *In* HIV Advances in Research and Therapy. Vol. 7. T.C. Merigan, D.P. Bolognesi, J. Feinberg, P.A. Volberding, and F. Wong-Staal, editors. Cliggott Communications, Greenwich, CT. 3–12.
2. Bukrinsky, M., S. Haggerty, M.P. Dempsey, N. Sharova, A. Adzhubei, L. Spitz, P. Lewis, D. Goldfarb, M. Emerman, and M. Stevenson. 1993. A nuclear localization signal within HIV-1 matrix protein that governs infection of non-dividing cells. *Nature*. 365:666–669.
3. Bukrinsky, M.I., N. Sharova, T.L. McDonald, T. Pushkarskaya, W.G. Tarpley, and M. Stevenson. 1993. Association of integrase, matrix, and reverse transcriptase antigens of human immunodeficiency virus type 1 with viral nucleic acids following acute infection. *Proc. Natl. Acad. Sci. USA*. 90:6125–6129.
4. Karageorgos, L., P. Li, and C. Burrell. 1993. Characterization of HIV replication complexes early after cell-to-cell infection. *AIDS Res. Hum. Retroviruses*. 9:817–823.
5. Heinzinger, N., M. Bukrinsky, S. Haggerty, A. Ragland, M.-A. Lee, V. Kewalramani, H. Gendelman, L. Ratner, M. Stevenson, and M. Emerman. 1994. The Vpr protein of human immunodeficiency virus type 1 influences nuclear localization of viral nucleic acids in nondividing host cells. *Proc. Natl. Acad. Sci. USA*. 91:7311–7315.
6. Gallay, P., S. Swingler, C. Aiken, and D. Trono. 1995. HIV-1 infection of nondividing cells: C-terminal tyrosine phosphorylation of the viral matrix protein is a key regulator. *Cell*. 80:379–388.
7. Miller, M.D., C.M. Farnet, and F.D. Bushman. 1997. Human immunodeficiency virus type 1 preintegration complexes: studies of organization and composition. *J. Virol.* 71: 5382–5390.
8. Farnet, C.M., and W.A. Haseltine. 1991. Determination of viral proteins present in the human immunodeficiency virus type 1 preintegration complex. *J. Virol.* 65:1910–1915.
9. Stevenson, M. 1996. Portals of entry: uncovering HIV nuclear transport pathways. *Trends Cell Biol*. 6:9–15.
10. Bukrinskaya, A.G., A. Ghorpade, N.K. Heinzinger, T.E. Smithgall, R.E. Lewis, and M. Stevenson. 1996. Phosphorylation-dependent human immunodeficiency virus type 1 infection and nuclear targeting of viral DNA. *Proc. Natl. Acad. Sci. USA*. 93:367–371.
11. Gheysen, D., E. Jacobs, F. de Foresta, C. Thiriart, M. Francotte, D. Thines, and M. De Wilde. 1989. Assembly and release of HIV-1 precursor Pr55*gag* virus-like particles from recombinant baculovirus-infected insect cells. *Cell*. 59:103–112.
12. Gottlinger, H.G., J.G. Sodroski, and W.A. Haseltine. 1989. Role of capsid precursor processing and myristoylation in morphogenesis and infectivity of human immunodeficiency virus type 1. *Proc. Natl. Acad. Sci. USA*. 86:5781–5785.
13. Bryant, M., and L. Ratner. 1990. Myristoylation-dependent replication and assembly of human immunodeficiency virus 1. *Proc. Natl. Acad. Sci. USA*. 87:523–527.
14. Jacqué, J.-M., A. Mann, H. Enslen, N. Sharova, B. Brichacek, R.J. Davis, and M. Stevenson. 1998. Modulation of HIV-1 infectivity by MAPK, a virion-associated kinase. *EMBO (Eur. Mol. Biol. Organ.) J.* 17:2607–2618.
15. Von Schwedler, U., R.S. Kornbluth, and D. Trono. 1994. The nuclear localization signal of the matrix protein of human immunodeficiency virus type 1 allows the establishment of infection in macrophages and quiescent T lymphocytes. *Proc. Natl. Acad. Sci. USA*. 91:6992–6996.
16. Freed, E.O., J.M. Orenstein, A.J. Buckler-White, and M.A. Martin. 1994. Single amino acid changes in the human immunodeficiency virus type 1 matrix protein block virus particle production. *J. Virol.* 68:5311–5320.
17. Fouchier, R.A., B.E. Meyer, J.H. Simon, U. Fischer, and M.H. Malim. 1997. HIV-1 infection of non-dividing cells: evidence that the amino-terminal basic region of the viral matrix protein is important for Gag processing but not for

post-entry nuclear import. *EMBO (Eur. Mol. Biol. Organ.) J.* 16:4531–4539.
18. Gallay, P., T. Hope, D. Chin, and D. Trono. 1997. HIV-1 infection of nondividing cells through the recognition of integrase by the importin/karyopherin pathway. *Proc. Natl. Acad. Sci. USA*. 94:9825–9830.
19. Vodicka, M.A., D.M. Koepp, P.A. Silver, and M. Emerman. 1998. HIV-1 Vpr interacts with the nuclear transport pathway to promote macrophage infection. *Genes Dev*. 12:175–185.
20. Nie, Z., D. Bergeron, R.A. Subbramanian, X.J. Yao, F. Checroune, N. Rougeau, and E.A. Cohen. 1998. The putative alpha helix 2 of human immunodeficiency virus type 1 Vpr contains a determinant which is responsible for the nuclear translocation of proviral DNA in growth-arrested cells. *J. Virol*. 72:4104–4115.
21. Subbramanian, R.A., A. Kessous-Elbaz, R. Lodge, J. Forget, X.J. Yao, D. Bergeron, and E.A. Cohen. 1998. Human immunodeficiency virus type 1 Vpr is a positive regulator of viral transcription and infectivity in primary human macrophages. *J. Exp. Med*. 187:1103–1111.
22. Subbramanian, R.A., X.J. Yao, H. Dilhuydy, N. Rougeau, D. Bergeron, Y. Robitaille, and E.A. Cohen. 1998. Human immunodeficiency virus type 1 Vpr localization: nuclear transport of a viral protein modulated by a putative amphipathic helical structure and its relevance to biological activity. *J. Mol. Biol*. 278:13–30.
23. Perotti, M.E., X. Tan, and D.M. Phillips. 1996. Directional budding of human immunodeficiency virus from monocytes. *J. Virol*. 70:5916–5921.
24. Bowerman, B., P.O. Brown, J.M. Bishop, and H.E. Varmus. 1989. A nucleoprotein complex mediates the integration of retroviral DNA. *Genes Dev*. 3:469–478.
25. Lori, F., V.F. di Marzo, A.L. de Vico, P. Lusso, M.S.J. Reitz, and R.C. Gallo. 1992. Viral DNA carried by human immunodeficiency virus type 1 virions. *J. Virol*. 66:5067–5074.
26. Trono, D. 1992. Partial reverse transcripts in virions from human immunodeficiency and murine leukemia viruses. *J. Virol*. 66:4893–4900.
27. Zhang, H., Y. Zhang, T. Spicer, D. Henrard, and B.J. Poiesz. 1995. Nascent human immunodeficiency virus type 1 reverse transcription occurs within an enveloped particle. *J. Virol*. 69: 3675–3682.
28. Preston, B.D., B.J. Poiesz, and L.A. Loeb. 1988. Fidelity of HIV-1 reverse transcriptase. *Science*. 242:1168–1171.
29. Bukrinsky, M.I., N. Sharova, M.P. Dempsey, T.L. Stanwick, A.G. Bukrinskaya, S. Haggerty, and M. Stevenson. 1992. Active nuclear import of human immunodeficiency virus type 1 preintegration complexes. *Proc. Natl. Acad. Sci. USA*. 89: 6580–6584.
30. Brown, P.O., B. Bowerman, H.E. Varmus, and J.M. Bishop. 1987. Correct integration of retroviral DNA *in vitro*. *Cell*. 49: 347–356.
31. Roe, T., T.C. Reynolds, G. Yu, and P.O. Brown. 1993. Integration of murine leukemia virus DNA depends on mitosis. *EMBO (Eur. Mol. Biol. Organ.) J.* 12:2099–2108.
32. Lewis, P.F., and M. Emerman. 1994. Passage through mitosis is required for oncoretroviruses but not for the human immunodeficiency virus. *J. Virol*. 68:510–516.
33. Pear, W.S., G.P. Nolan, M.L. Scott, and D. Baltimore. 1993. Production of high-titer helper-free retroviruses by transient transfection. *Proc. Natl. Acad. Sci. USA*. 90:8392–8396.
34. Peden, K., M. Emerman, and L. Montagnier. 1991. Changes in growth properties on passage in tissue culture of viruses derived from infectious molecular clones of HIV-1_{LAI}, HIV-1_{MAL}, and HIV-1_{ELI}. *Virology*. 185:661–672.
35. Kimpton, J., and M. Emerman. 1992. Detection of replication-competent and pseudotyped human immunodeficiency virus with a sensitive cell line on the basis of activation of an integrated beta-galactosidase gene. *J. Virol*. 66:2232–2239.
36. Bartz, S.R., M.E. Rogel, and M. Emerman. 1996. Human immunodeficiency virus type 1 cell cycle control: Vpr is cytostatic and mediates G_2 accumulation by a mechanism which differs from DNA damage checkpoint control. *J. Virol*. 70: 2324–2331.
37. Zack, J.A., S.J. Arrigo, S.R. Weitsman, A.S. Go, A. Haislip, and I.S.Y. Chen. 1990. HIV-1 entry into quiescent primary lymphocytes: molecular analysis reveals a labile, latent viral structure. *Cell*. 61:213–222.
38. Folks, T.M., D. Powell, M. Lightfoote, S. Koenig, A.S. Fauci, S. Benn, A. Rabson, D. Daugherty, H.E. Gendelman, M.D. Hoggan, et al. 1986. Biological and biochemical characterization of a cloned LEU-3^- cell surviving infection with the acquired immune deficiency syndrome retrovirus. *J. Exp. Med*. 164:280–290.
39. Myers, G., J. Berzofsky, B. Korber, R.F. Smith, and G.N. Pavlakis. 1993. Human retroviruses and AIDS 1993: a compilation and analysis of nucleic acid and amino acid sequences. Theoretical Biology and Biophysics Group T-10, Los Alamos National Laboratory, Los Alamos, NM.
40. Lochrie, M.A., S. Waugh, D.G. Pratt, Jr., J. Clever, T.G. Parslow, and B. Polisky. 1997. In vitro selection of RNAs that bind to the human immunodeficiency virus type-1 gag polyprotein. *Nucleic Acids Res*. 25:2902–2910.
41. Geppert, T.D., and P.E. Lipsky. 1991. Association of various T cell-surface molecules with the cytoskeleton. Effect of cross-linking and activation. *J. Immunol*. 146:3298–3305.
42. Kinch, M.S., J.L. Strominger, and C. Doyle. 1993. Cell adhesion mediated by CD4 and MHC class II proteins requires active cellular processes. *J. Immunol*. 151:4552–4561.
43. Miranda, A.F., G.C. Godman, A.D. Deitch, and S.W. Tanenbaum. 1974. Action of cytochalasin D on cells of established lines. I. Early events. *J. Cell Biol*. 61:481–500.
44. Brown, S.S., and J.A. Spudich. 1981. Mechanism of action of cytochalasin: evidence that it binds to actin filament ends. *J. Cell Biol*. 88:487–491.
45. Dellagi, K., and J.C. Brouet. 1982. Redistribution of intermediate filaments during capping of lymphocyte surface molecules. *Nature*. 298:284–286.
46. De Brabander, M.J., R.M. Van de Veire, F.E. Aerts, M. Borgers, and P.A. Janssen. 1976. The effects of methyl (5-(2-thienylcarbonyl)-1H-benzimidazol-2-yl) carbamate, (R 17934; NSC 238159), a new synthetic antitumoral drug interfering with microtubules, on mammalian cells cultured in vitro. *Cancer Res*. 36:905–916.
47. Rey, O., J. Canon, and P. Krogstad. 1996. HIV-1 gag protein associates with F-actin present in microfilaments. *Virology*. 220:530–534.
48. Coffin, J.M. 1996. *Retroviridae*: the viruses and their replication. *In* Fields Virology, 3rd ed. Vol. 2. B.N. Fields, D.M. Knipe, P.M. Howley, R.M. Chanock, J.L. Melnick, T.P. Monath, B. Roizman, and S.E. Straus, editors. Lippincott-Raven Publishers, Philadelphia. 1767–1847.
49. Mitchison, T.J., and L.P. Kramer. 1996. Actin-based cell motility and cell locomotion. *Cell*. 84:371–379.
50. Adelstein, R.S. 1983. Regulation of contractile proteins by

phosphorylation. *J. Clin. Invest.* 72:1863–1866.
51. Wilson, A.K., G. Gorgas, W.D. Claypool, and P. de Lanerolle. 1991. An increase or a decrease in myosin II phosphorylation inhibits macrophage motility. *J. Cell Biol.* 114:277–283.
52. Jay, P.Y., P.A. Pham, S.A. Wong, and E.L. Elson. 1995. A mechanical function of myosin II in cell motility. *J. Cell Sci.* 108:387–393.
53. Saitoh, M., T. Ishikawa, S. Matsushima, M. Naka, and H. Hidaka. 1987. Selective inhibition of catalytic activity of smooth muscle myosin light-chain kinase. *J. Biol. Chem.* 262: 7796–7801.
54. Nakanishi, S., K. Yamada, K. Iwahashi, K. Kuroda, and H. Kase. 1990. KT5926, a potent and selective inhibitor of myosin light-chain kinase. *Mol. Pharmacol.* 37:482–488.
55. Stein, B.S., S.D. Gowda, J.D. Lifson, R.C. Penhallow, K.G. Bensch, and E.G. Engleman. 1987. pH-independent HIV entry into CD4-positive T cells via virus envelope fusion to the plasma membrane. *Cell.* 49:659–668.
56. Aiken, C. 1997. Pseudotyping human immunodeficiency virus type 1 (HIV-1) by the glycoprotein of vesicular stomatitis virus targets HIV-1 entry to an endocytic pathway and suppresses both the requirement for Nef and the sensitivity to cyclosporin A. *J. Virol.* 71:5871–5877.
57. Iyengar, S., J.E.K. Hildreth, and D.H. Schwartz. 1998. Actin-dependent receptor colocalization required for human immunodeficiency virus entry into host cells. *J. Virol.* 72: 5251–5255.
58. Moyer, S.A., S.C. Baker, and J.L. Lessard. 1986. Tubulin: a factor necessary for the synthesis of both Sendai virus and vesicular stomatitis virus RNAs. *Proc. Natl. Acad. Sci. USA.* 83: 5405–5409.
59. Hill, V.M., and D.F. Summers. 1990. A minor microtubule-associated protein is responsible for the stimulation of vesicular stomatitis virus transcription in vitro. *J. Virol.* 71:289–298.
60. Huang, Y.T., R.R. Romito, B.P. De, and A.K. Banerjee. 1993. Characterization of the *in vitro* system for the synthesis of mRNA from human respiratory syncytial virus. *Virology.* 193:862–867.
61. De, B.P., A. Lesoon, and A.K. Banerjee. 1991. Human parainfluenza virus type 3 transcription in vitro: role of cellular actin in mRNA synthesis. *J. Virol.* 65:3268–3275.
62. De, B.P., A.L. Burdsall, and A.K. Banerjee. 1993. Role of cellular actin in human parainfluenza virus type 3 genome transcription. *J. Biol. Chem.* 268:5703–5710.
63. Gupta, S., B.P. De, J.A. Drazba, and A.K. Banerjee. 1998. Involvement of actin microfilaments in the replication of human parainfluenza virus type 3. *J. Virol.* 72:2655–2662.
64. Hottiger, M., K. Gramatikoff, O. Georgiev, C. Chaponnier, W. Schaffner, and U. Hubscher. 1995. The large subunit of HIV-1 reverse transcriptase interacts with beta-actin. *Nucleic Acids Res.* 23:736–741.
65. Arts, E.J., and M.A. Wainberg. 1996. Human immunodeficiency virus type 1 reverse transcriptase and early events in reverse transcription. *Adv. Virus Res.* 46:97–163.
66. Woodard, M.H., W.A. Dunn, R.O. Laine, M. Malandro, R. McMahon, O. Simell, E.R. Block, and M.S. Kilberg. 1994. Plasma membrane clustering of the system y+ (CAT-1) amino acid transporter as detected by immunohistochemistry. *Am. J. Physiol.* 266:E817–E824.
67. Kizhatil, K., and L.M. Albritton. 1997. Requirements for different components of the host cell cytoskeleton distinguish ecotropic murine leukemia virus entry via endocytosis from entry via surface fusion. *J. Virol.* 71:7145–7156.
68. Pearce-Pratt, R., D. Malamud, and D.M. Phillips. 1994. Role of the cytoskeleton in cell-to-cell transmission of human immunodeficiency virus. *J. Virol.* 68:2898–2905.
69. 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.
70. Ott, D.E., L.V. Coren, B.P. Kane, L.K. Busch, D.G. 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.
71. Karczewski, M.K., and K. Strebel. 1996. Cytoskeleton association and virion incorporation of the human immunodeficiency virus type 1 Vif protein. *J. Virol.* 70:494–507.
72. Saib, A., F. Puvion-Dutilleul, M. Schmid, J. Peries, and H. de The. 1997. Nuclear targeting of incoming human foamy virus Gag proteins involves a centriolar step. *J. Virol.* 71: 1155–1161.
73. Sodeik, B., M.W. Ebersold, and A. Helenius. 1997. Microtubule-mediated transport of incoming herpes simplex virus 1 capsids to the nucleus. *J. Cell Biol.* 136:1007–1021.