JOURNAL OF VIROLOGY, June 2008, p. 5860–5868 Vol. 82, No. 12
0022-538X/08/$08.00+0 doi:10.1128/JVI.00076-08

The LEM Domain Proteins Emerin and LAP2α Are Dispensable for Human Immunodeficiency Virus Type 1 and Murine Leukemia Virus Infections▽†

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Received 11 January 2008/Accepted 31 March 2008

The human nuclear envelope proteins emerin and lamina-associated polypeptide 2α (LAP2α) have been proposed to aid in the early replication steps of human immunodeficiency virus type 1 (HIV-1) and murine leukemia virus (MLV). However, whether these factors are essential for HIV-1 or MLV infection has been questioned. Prior studies in which conflicting results were obtained were highly dependent on RNA interference-mediated gene silencing. To shed light on these contradictory results, we examined whether HIV-1 or MLV could infect primary cells from mice deficient for emerin, LAP2α, or both emerin and LAP2α. We observed HIV-1 and MLV infectivity in mouse embryonic fibroblasts (MEFs) from emerin knockout, LAP2α knockout, or emerin and LAP2α double knockout mice to be comparable in infectivity to wild-type littermate-derived MEFs, indicating that both emerin and LAP2α were dispensable for HIV-1 and MLV infection of dividing, primary mouse cells. Because emerin has been suggested to be important for infection of human macrophages by HIV-1, we also examined HIV-1 transduction of macrophages from wild-type mice or knockout mice, but again we did not observe a difference in susceptibility. These findings prompted us to reexamine the role of human emerin in supporting HIV-1 and MLV infection. Notably, both viruses efficiently infected human cells expressing high levels of dominant-negative emerin. We thus conclude that emerin and LAP2α are not required for the early replication of HIV-1 and MLV in mouse or human cells.

Viruses that enter or egress from the host cell nucleus are influenced by proteins in the nuclear milieu and have devised strategies to overcome and exploit innate barriers in order to sustain and spread infection (36). The nuclear architecture of eukaryotes is maintained by a network of intermediate filament proteins, the lamins, located beneath the inner nuclear membrane known as the lamina (5). In addition to providing mechanical strength and shape to nuclei, lamins support both the nuclear pores and other lamin-associated proteins. Some of these other lamina-associated proteins include a family defined by a ~40-residue motif called the LEM domain (9, 15). The LEM domain family consists of several proteins, including emerin (3), lamina-associated polypeptide 2 (LAP2) (7), MAN1 (18), otefin (8), Lem3 (13), and Lem2 (4). The LEM domain is essential for interaction of these laminar proteins with the host chromatin protein barrier-to-autointegration factor (BAF) (14, 22, 26). BAF is believed to be a component of human immunodeficiency virus type 1 (HIV-1) and murine leukemia virus (MLV) preintegration complexes (PICs) and to facilitate productive infection by preventing intramolecular integration of the viral DNA into itself (autointegration) and promoting intermolecular integration into extraneous (host) DNA (6, 16, 17, 29).

Recent studies have suggested that two LEM domain proteins, emerin and LAP2α, promote HIV-1 and MLV infectivity by enhancing chromatin association and integration of viral cDNA, likely through BAF interactions (11, 30). Depletion of emerin from human cells impaired infection by HIV-1 displaying wild-type Env or the G protein of vesicular stomatitis virus (VSV-G), whereas depletion of LAP2α reduced infection by HIV-1 displaying wild-type Env, MLV bearing wild-type Env, or MLV pseudotyped with VSV-G (11). Emerin, in particular, appeared to be critical for HIV-1 infection of primary macrophages. In addition, it has been reported that mouse cells depleted of LAP2α are diminished in their capacity to support MLV replication (30). However, the requirement of these factors for HIV-1 or MLV infection has been questioned (27).

Because these discordant results were generated through similar RNA interference (RNAi)-based techniques, we sought to investigate the roles of emerin and LAP2α in retroviral infection using alternative approaches. Studies of the HIV-1 IN binding protein, LEDGF, revealed that even minute quantities of a viral cofactor remaining after RNAi-mediated depletion are sufficient to permit efficient virus integration (19). We therefore examined whether HIV-1 or MLV could infect primary cells from mice whose genes that encode emerin, LAP2α, or both emerin and LAP2α were ablated. In contrast to studies implicating roles for emerin and LAP2α in

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† Supplemental material for this article may be found at http://jvi.asm.org/.

▽ Published ahead of print on 9 April 2008.

retroviral infection, we did not observe either protein to be essential for HIV-1 or MLV infection of mouse cells. In addition, a dominant-negative form of emerin did not impair HIV-1 or MLV infectivity in human cells. While these studies do not rule out an interaction of HIV-1 or MLV with nuclear lamin proteins during infection, they suggest that these interactions may not be critical for provirus establishment.

MATERIALS AND METHODS

Generation of knockout mice. The emerin-deficient ($Emd^{-/-}$) mice have been described previously (24). The LAP2α-deficient ($Tmpo^{-/-}$) mice have also been described elsewhere (N. Naetar et al., submitted for publication). The $Emd^{-/-}$ and $Tmpo^{-/-}$ mice were intercrossed to obtain $Emd^{-/-}$ $Tmpo^{-/-}$ double homozygous mice. All mice were maintained in accordance with the procedures outlined in the *Guide for the Care and Use of Laboratory Animals* of the NCI-Frederick ACUC Guidelines and Policies Committee.

Isolation of mouse embryonic fibroblasts and immunofluorescence analysis. Mouse embryos were harvested at day 13 postconception, eviscerated, and homogenized in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 0.1 mg/ml DNase I (Sigma), and 0.5 mg/ml collagenase (Sigma). After 30 min of incubation, cells were washed in DMEM containing 10% FBS and 100 U/ml penicillin and 0.1 mg/ml streptomycin and plated to establish the primary fibroblast lines. Individual mouse embryonic fibroblast (MEF) lines were expanded and genotyped by Southern blot analysis as described elsewhere (24; Naetar et al., submitted). Cells used in infection experiments were at passage 4 to 5. For immunofluorescence analysis, MEFs were grown on microscope slide coverslips, fixed with 4% paraformaldehyde, stained with antibodies specific for either emerin (Novocastra, United Kingdom) or LAP2α (35) and then with secondary antibodies conjugated to Alexa 568 (Molecular Probes, Eugene, OR), and counterstained with 4′,6′-diamidino-2-phenylindole. After immunolabeling, coverslips were mounted in Vectashield mounting medium (Vector Laboratories, Burlingame, CA) and visualized using a Zeiss Axiophot inverted microscope.

Genotyping (RT-PCR). Mice of known genetic backgrounds were used to isolate macrophages or used as breeding pairs to obtain MEFs. Emerin and LAP2α genotypes were further characterized in isolated cells by reverse transcription-PCR (RT-PCR). Total cellular RNA from the mouse macrophages used in Fig. 4, below, and the MEFs used in Fig. 2 and 3, below, were extracted with the RNeasy kit (Qiagen). Each sample was normalized for total RNA by spectrophotometric analysis and was subjected to a single round of RT-PCR using oligo(dT) oligonucleotides and the First Strand cDNA synthesis kit (Roche) to generate cDNA. The cDNA was subjected to 30 rounds of PCR using the following specific primers: for *Emd* (NM_000117), forward primer 5′-TTG TCTGCCATGGACGACTATGC-3′ and reverse primer 5′-TAAGAGCTGCT CTAAAACCAATACC-3′, which amplify a 914-bp product containing sequence from exon 1 through exon 6; for *Tmpo* (U09086), forward primer 5′-TGGAGG GAAGAGTAGAGCTCAG-3′ and reverse primer 5′-GTTCGGATCCAGGTG TATTCTG-3′, which amplify a 373-bp product within exon 4; for *Actb* (actin, beta; NM_007393), forward primer 5′-TGAACCCTAAGGCCAACCGTG-3′ and reverse primer 5′-GCTCATAGCTCTTCTCCAGGG-3′; for *Hprt1* (hypoxanthine guanine phosphoribosyl transferase 1; NM_013556), forward primer 5′-CCTAAGATGAGCGCAAGTTGAA-3′ and reverse primer 5′-CCACAGG ACTAGAACACCTGCTAA-3′. Following PCR amplification, the samples were run on 1.0% agarose to confirm genotype, i.e., the absence or presence of mRNA.

Cell culture and antiviral drugs. The 293T and NIH 3T3 cell lines were maintained in DMEM supplemented with 10% FBS, 100 U/ml penicillin, and 0.1 mg/ml streptomycin. The MEFs were maintained in DMEM supplemented with 10% FBS, 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 0.1 mM β-mercaptoethanol. The primary mouse macrophages were maintained in RPMI 1640 medium supplemented with 10% FBS, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 0.1 mM β-mercaptoethanol, 0.3% sodium bicarbonate, and 0.25% conditioned medium containing granulocyte-macrophage colony-stimulating factor (gift of Derya Unutmaz, NYU). The nonnucleoside reverse transcriptase inhibitor efavirenz (EFV) was obtained from the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH.

Plasmids. The HIV-CMV-GFP (HCG) plasmid was constructed from the HIV-EGFP plasmid (32) using the unique 5′ NotI and 3′ XhoI at the ends of the enhanced green fluorescent protein (EGFP) coding sequence, thus replacing EGFP with cytomegalovirus (CMV)-EGFP. This plasmid contains deletions in *vif*, *vpr*, *env*, and *nef*. The NLdE-CMV-GFP (NLdECG) plasmid was constructed from wild-type pNL4-3 to contain the same *env* gene deletion as the pLai3ΔenvGFP3 plasmid (38). The NLdECG plasmid contains deletions in *env* and *nef*. The integrase catalytic site mutant, IN-D116N, was generated by PCR mutagenesis and confirmed by sequencing.

The cDNA for full-length human *Emd* (GenBank accession number NM_000117) was obtained from Open Biosystems. The two dominant-negative LEM mutants were made by site-directed mutagenesis. The delta-LEM mutant is deleted for the entire LEM domain coding sequence (coding for amino acids 3 to 44) as described by Jacque and Stevenson (11), while the m24-LEM mutant described by Lee and colleagues has alanine substitutions at amino acid positions 24, 25, 26, and 27 (14). Both wild-type and LEM mutant emerin were inserted into the pIRES-puro3 expression vector (Clontech) using the AgeI and BamHI sites.

Virus stocks. HIV-1 virus stocks were produced by DNA transfection on monolayer cultures of 293T cells grown in 10-cm culture dishes using Lipofectamine 2000 transfection reagent (Invitrogen). Each 10-cm dish was cotransfected with 18 μg of viral vector and 6 μg of p-L-VSV-G (1) or pSV-A-MLV (amphotropic) *env* (12) expression plasmids. Moloney MLV stocks were produced by DNA transfections of 293T cells grown in six-well plates using the calcium phosphate DNA precipitation method. Each cell monolayer (well) was cotransfected with 5 μg of pMIGR1 (25), 2.5 μg of pJK3 (1), 1 μg of pCMV-Tat, and 2 μg of p-L-VSV-G plasmids (1). Culture supernatants from the 293T cells were collected 48 h posttransfection, clarified by low-speed centrifugation (1,000 × *g*, 10 min), and filtered through 0.2-μm-pore-size sterile filters. For the HIV-1 vectors, the clarified supernatants were analyzed for p24 antigen concentration by enzyme-linked immunosorbent assay (Beckman-Coulter Inc.).

Infection assays. Infectivity for all virus stocks was initially determined by titration on NIH 3T3 cells. Briefly, virus-containing supernatants were serially diluted (threefold dilutions) and used to infect monolayer cultures of NIH 3T3 cells plated on the previous day at 20,000 cells/well in 12-well tissue culture plates (Corning). At 48 h postinfection, the cells were trypsinized and resuspended in phosphate-buffered saline (PBS) containing 2% FBS. The expression of GFP following infection by the different HIV-1 and Moloney MLV vectors was measured by fluorescence-activated cell sorter (FACS) analysis (FACSCalibur; Becton Dickinson). Using the titration curves obtained from the NIH 3T3 infections, set doses of VSV-G- or Ampho Env-pseudotyped HCG vector (HIV/VSV-G and HIV/Ampho, respectively) or VSV-G Env-pseudotyped MIGR1 vector (MLV/VSV-G) were used to infect the MEFs that were plated on the previous day at 20,000 cells/well in 12-well tissue culture plates (Corning). The infection of MEFs by the HIV-1 and MLV vectors was measured by GFP expression at 48 h postinfection using FACS analysis, as described above. The percent infected cells represents the percentage of GFP-positive cells in the cell population.

The infection efficiencies of HIV/VSV-G on macrophages from $Emd^{-/-}$ or $Tmpo^{-/-}$ knockout and $Emd^{-/-}$ $Tmpo^{-/-}$ double knockout mice compared to wild-type littermate controls were examined. The peritoneal cavity of mice was injected with 2 ml of 3% thioglycolate in PBS. At 3 days after thioglycolate injection, the thioglycolate-responsive macrophage populations were harvested in 5 ml ice-cold Hanks' balanced salt solution and washed once with macrophage culture medium. The macrophages were plated at a cell density of 100,000 cells/well in 24-well low-attachment plates (Corning). After overnight incubation at 37°C, the cells were infected with a set dose of VSV-G Env-pseudotyped NLdECG vector (HIV/VSV-G). At 60 h postinfection, the cells were taken off the plate, washed once with 10 ml PBS containing 2% FBS, and kept on ice for subsequent steps. The cells were resuspended in 200 μl of PBS containing 2% FBS. The rat anti-mouse CD16/CD32 (FcγIII/II receptor) monoclonal antibody (mouse BD Fc block) was used to block the Fc-mediated binding of antibodies to mouse Fcγ receptor-bearing macrophages as recommended by the manufacturer (BD Pharmingen). After blocking the Fcγ receptors, the cells were stained with either the mouse macrophage-specific anti-F4/80–allophycocyanin or the isotype control antibody (Caltag Laboratories). F4/80 is a macrophage-restricted cell surface glycoprotein (23). The cells were washed twice after staining for 30 min, resuspended in 0.5 ml PBS containing 2% FBS, and analyzed by FACS. The typical purity of isolated macrophages by F4/80 staining is shown under the M1 gate in Fig. S1 in the supplemental material. The percent infected cells was measured as the percentage of GFP-positive cells in the F4/80-positive population.

The effect of dominant-negative emerin was examined using cells expressing wild-type and mutant forms of emerin. To measure infection by HIV-1 and MLV, 293T cells were transiently transfected with pIRES-puro3, pEmerin(WT)-IRES-puro, pEmerin(delta-LEM)-IRES-puro, or pEmerin(m24-LEM)-IRES-puro in a six-well plate. Cells were cotransfected with pDsRed-monomer-N1

(Clontech) at a threefold-lower molar amount. The cells were replated 1 day posttransfection at 1×10^6 cells/well in a fresh 6-well plate for obtaining Western blot cell lysates and at 4×10^4 cells/well in a 24-well plate for infection with HIV/VSV-G or MLV/VSV-G virus. On day 2 posttransfection, the cells from the six-well plate were collected for Western blotting. The cells in the 24-well plate were infected with a set dose of previously titrated HIV/VSV-G or MLV/VSV-G virus. At 48 h from the time of infection the cells were trypsinized, resuspended in PBS containing 2% FBS, and analyzed by FACS. The percent infected cells was measured as the percentage of GFP-positive cells in the DsRed-positive population.

RESULTS

Analysis of cells from knockout mice. The emerin- and LAP2α-deficient mice have been described previously (24; Naetar et al., submitted). Deletion of *Emd* exons 2 to 5 eliminates all but the first exon of the X-linked gene, resulting in a complete loss of emerin synthesis (24). By contrast, the *Tmpo* knockout used in this study was restricted to exon 4, which enables expression of all LAP2 isoforms except for LAP2α (Naetar et al., submitted). Removal of emerin did not impair LAP2α production or subcellular distribution in MEFs, and the elimination of LAP2α had no effect on emerin levels or distribution (Fig. 1A and data not shown). In addition, the overall nuclear morphologies in the single and double knockout cells were grossly similar to wild-type cells.

For subsequent infection experiments, control cells expressing wild-type proteins were obtained from littermates of knockout mice to minimize possible maternal effects and ensure matched developmental times. Mouse macrophage and MEF genotypes were verified by RT-PCR. The macrophages and MEFs from wild-type littermate controls showed the presence of both *Emd* and *Tmpo* mRNA, and single knockout mice for either *Emd* or *Tmpo* showed an absence of the respective mRNA, while those from the double knockout mice showed an absence of both mRNAs (Fig. 1B). The RT-PCR for β-actin and HPRT mRNA confirmed the presence of cellular RNA in all the samples.

HIV-1 and MLV can infect MEFs lacking either emerin or LAP2α. RNAi studies by Jacque and Stevenson suggested that emerin regulated HIV-1 infection of immortalized (dividing) human cells after nuclear entry (11). These results have been questioned by Shun and colleagues employing both RNAi in human cells and one *Emd* knockout male MEF line that we provided to assay sensitivity to HIV-1 or MLV infection (27). The underlying reasons for the different experimental outcomes are unclear. In the studies with the mouse cells, we examined whether other *Emd* knockout lines were also susceptible to retroviral infection or if other LEM-domain containing proteins played a more prominent role in the infection of mouse cells. To test whether emerin is required for HIV-1 or MLV infection of dividing mouse cells, we infected MEFs that do not have a functional *Emd* gene with HIV/VSV-G, HIV/Ampho, or MLV/VSV-G. HIV/VSV-G was used to infect emerin-deficient MEFs from both male and female mice (−/Y and −/−, respectively) along with MEFs from sex-matched wild-type littermate controls (+/Y and +/+, respectively). Infection of these MEF lines with an equal dose of HIV/VSV-G did not result in significantly different infection levels (Fig. 2A, left panel). Also, the HIV/Ampho and MLV/VSV-G viruses showed similar infectivity on emerin-deficient (−/−) and wild-type (+/+) MEFs (middle and right panels, respectively). Infections with greater or lesser titers of virus did not result in any observable differences in susceptibility between the different wild-type and knockout lines (data not shown).

LAP2α has been associated with HIV-1 and MLV infections of immortalized human and mouse cell lines (11, 30). It is possible that the presence of LAP2α in the emerin-deficient MEFs enabled efficient retroviral infection. To examine the role of LAP2α in both HIV-1 and MLV infection, we infected MEFs from mice lacking exon 4 of the *Tmpo* gene with HIV/VSV-G, HIV/Ampho, or MLV/VSV-G. Infection of the LAP2α-deficient MEFs (−/−) with HIV/VSV-G and HIV/Ampho was similar to that of MEFs from wild-type littermate controls (+/+) (Fig. 2B, left and middle panels, respectively). In MLV/VSV-G infections, while some LAP2α-deficient MEF lines showed wild-type-like infection, other LAP2α-deficient MEF lines showed an approximately 30% reduced infection (right panel).

HIV-1 and MLV can infect MEFs lacking both emerin and LAP2α. Because both emerin and LAP2α are LEM domain proteins, we determined whether these proteins could be functionally redundant in supporting retroviral infection. To evaluate whether emerin and LAP2α are important for HIV-1 and MLV infection, we examined the infectivity of HIV/VSV-G, HIV/Ampho, or MLV/VSV-G on MEFs from *Emd* and *Tmpo* double knockout mice. Infection of the emerin- and LAP2α-deficient MEFs (−/−) with HIV/VSV-G and HIV/Ampho was comparable to that of MEFs from wild-type littermate controls (+/+) (Fig. 3A, left and middle panels, respectively). MLV/VSV-G infectivity of the double knockout MEFs showed an approximately 30% reduced infection compared to wild-type MEFs (right panel). Although the susceptibility of this line was consistently lower, another double knockout line showed similar infectivity to wild-type cells in other experiments (data not shown).

Because it has been suggested that emerin and LAP2α regulate the fate of viral DNA (vDNA) in the nucleus, potentially facilitating interactions with chromatin (11), it was conceivable that the positive infections scored in the absence of the LEM domain-containing proteins actually reflected stable forms of vDNA that were transcriptionally active but failed to integrate. To test whether *Emd* knockout, *Tmpo* knockout, or *Emd* and *Tmpo* double knockout MEFs enriched unintegrated vDNAs to a greater extent than wild-type control MEFs, we infected these MEFs with wild-type HIV/VSV-G or $HIV^{IN\text{-}D116N}$/VSV-G (IN catalytic site mutation, D116N). As previously seen, infecting *Emd* or *Tmpo* knockout, *Emd* and *Tmpo* double knockout, and wild-type MEFs with an equal dose of HIV/VSV-G (200 ng p24/CA) resulted in similar infection levels (Fig. 3B). Notably, although the infectivity of $HIV^{IN\text{-}D116N}$/VSV-G virus in the MEFs was reduced approximately 100-fold compared to wild-type HIV/VSV-G, these infection levels were similar on *Emd* or *Tmpo* knockout, *Emd* and *Tmpo* double knockout, and wild-type MEFs. Because increased reporter gene expression by IN-defective HIV-1 was not apparent in knockout MEFs, wild-type HIV-1 infection detected in the absence of emerin or LAP2α remained integration dependent. The inability to detect infection of MEFs by wild-type HIV/VSV-G or $HIV^{IN\text{-}D116N}$/VSV-G in the presence of the nonnucleoside reverse transcriptase inhibitor EFV further con-

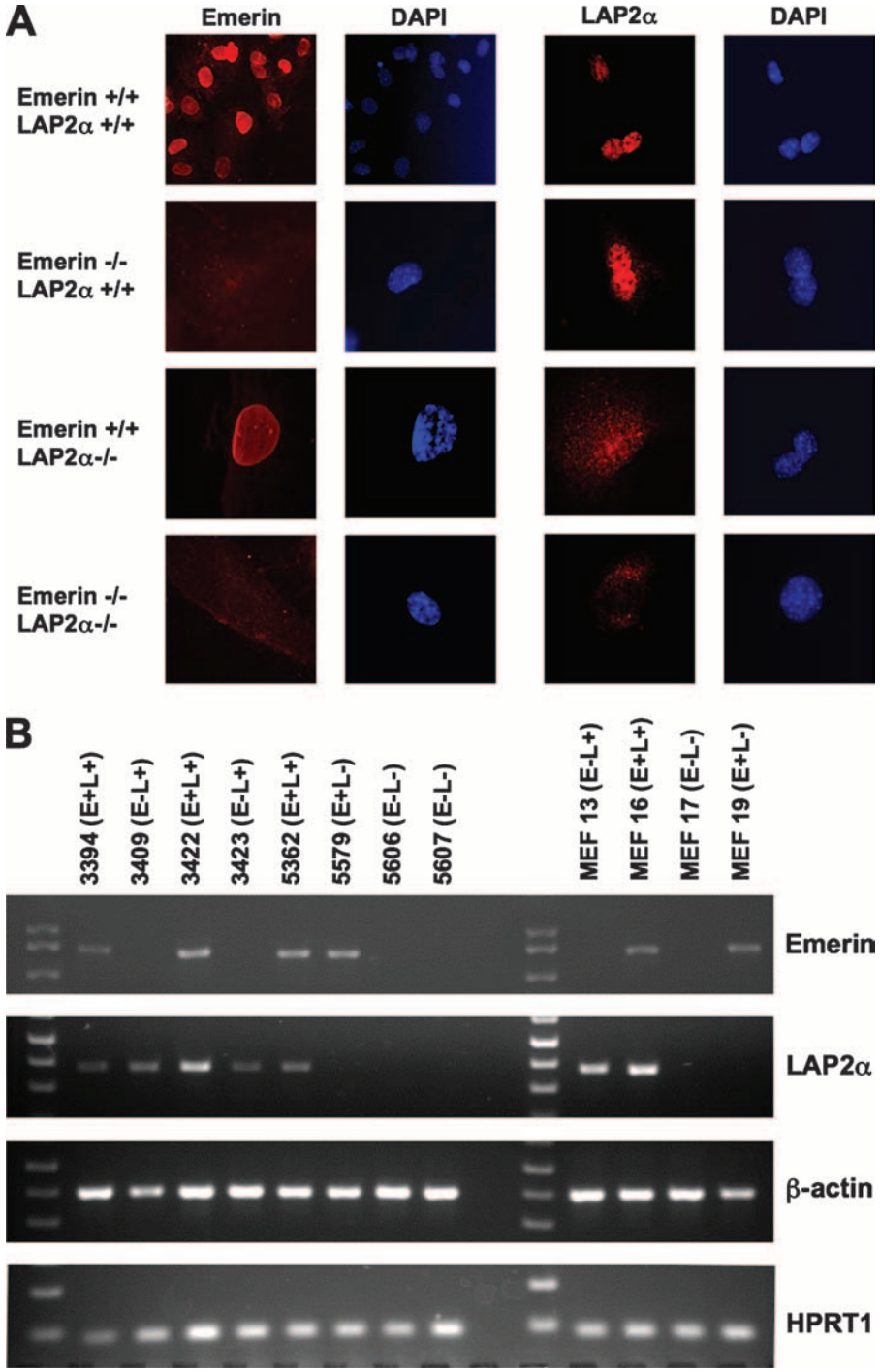

FIG. 1. Emerin- and LAP2α-deficient mouse cells. (A) The expression of emerin and LAP2α in fibroblasts derived from the single and double homozygote mice was analyzed by immunofluorescence. Staining with an emerin antibody showed the absence of the perinuclear staining pattern in all $Emd^{-/-}$ cells. Staining with LAP2α antibody showed a nucleoplasmic staining pattern that was absent in $Lap2\alpha^{-/-}$ cells, although a low level of background staining was evident with this antibody (35). (B) Genotyping of primary macrophages and MEFs by RT-PCR. Total cellular RNA from mouse macrophages used in Fig. 4, below, and MEFs used in Fig. 2 and 3, below, was subjected to RT-PCR to generate cDNA. Using primers specific for emerin, LAP2α, β-actin, and the HPRT-encoding mRNA sequence, the cDNA was subjected to 30 rounds of PCR. After PCR amplification, the samples were run on 1.0% agarose to confirm genotypes. β-Actin and HPRT were amplified as controls to demonstrate the presence of RNA in the cell extracts.

firmed that the infection observed was real (and not pseudo-infection by VSV-G vesicles containing GFP).

HIV-1 can infect macrophages from mice lacking emerin or LAP2α. The LEM domain-containing nuclear envelope proteins emerin and LAP2α have also been implicated in HIV-1 transduction of nondividing primary human macrophages (11). To test the role of emerin and LAP2α in HIV-1 infection, we obtained macrophages from *Emd* or *Tmpo* knockout, *Emd* and

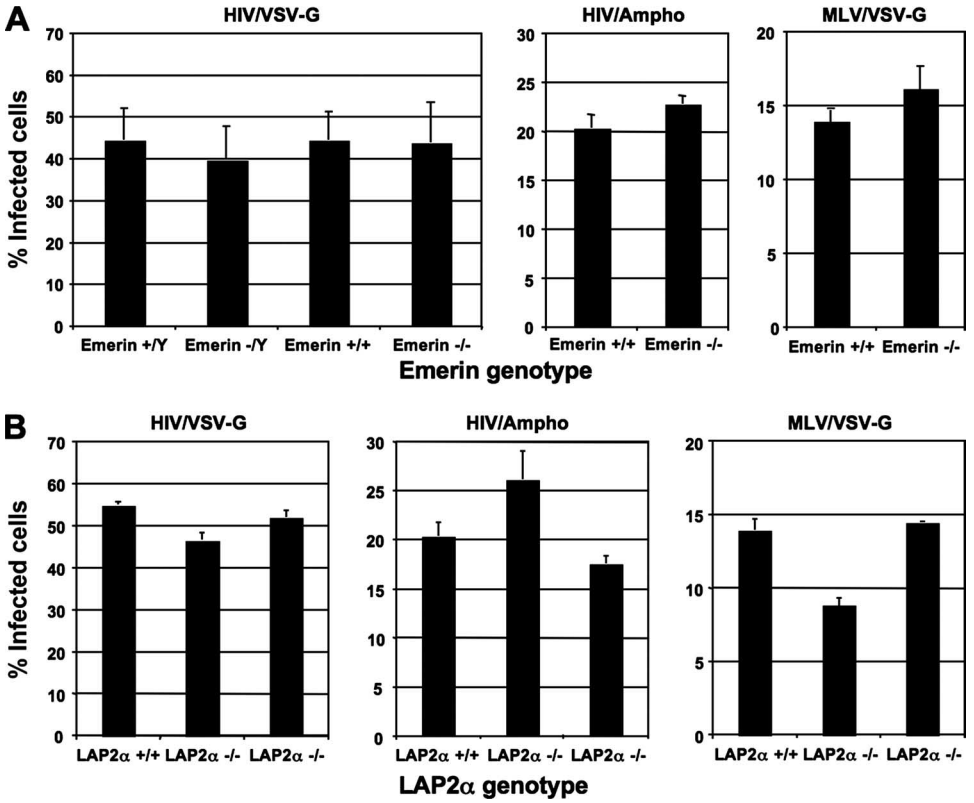

FIG. 2. HIV-1 and MLV can infect MEFs that are knocked out for either emerin or LAP2α expression. (A) Infection efficiency of HIV/VSV-G, HIV/Ampho, or MLV/VSV-G was examined in *Emd* (emerin) knockout MEFs compared to sex-matched, wild-type littermate control MEFs. The percent infectivity represents the percentage of GFP-expressing MEFs 48 h postinfection with HIV-1 or MLV vectors. Each data set represents the mean of three independent experiments, and error bars indicate standard deviations. (B) Infection efficiency of HIV/VSV-G, HIV/Ampho, or MLV/VSV-G was also examined in LAP2α knockout MEFs compared to wild-type littermate control MEFs. The percent infectivity represents the percentage of GFP-expressing MEFs 48 h postinfection with HIV-1 or MLV vectors. Each data set represents the mean of three separate wells of infected cells, and error bars indicate standard deviations.

Tmpo double knockout, and wild-type littermate control mice. The genotypes of these mice were confirmed by RT-PCR from lysates of the macrophages used in the infectivity studies (Fig. 1B).

The ability of HIV/VSV-G to infect macrophages from these *Emd* or *Tmpo* knockout, *Emd* and *Tmpo* double knockout, and wild-type littermate control mice was tested. The percentage of GFP-positive cells in the population expressing the mouse macrophage-specific F4/80 glycoprotein was used to determine the percent infected cells. The macrophages from the wild-type control mice numbers 5362 (female; $Emd^{+/+}$ $LAP2\alpha^{+/+}$), 3394 (male; $Emd^{+/y}$ $LAP2\alpha^{+/+}$), and 3422 (female; $Emd^{+/-}$ $LAP2\alpha^{+/+}$) showed an approximate infectivity of 4.5% (Fig. 4B, lanes 1, 2, and 3). The macrophages from the *Emd*-deficient mice, numbers 3409 (male; $Emd^{-/y}$ $LAP2\alpha^{+/+}$) and 3423 (female; $Emd^{-/-}$ $LAP2\alpha^{+/+}$), showed an infectivity of 5.0% (lanes 4 and 5). The macrophages from the *Tmpo*-deficient mouse, number 5579 (male; $Emd^{+/y}$ $LAP2\alpha^{-/-}$), had an infectivity of approximately 4.2% (lane 6). The macrophages from the *Emd* and *Tmpo* double knockout mice, numbers 5606 and 5607 (female; $Emd^{-/-}$ $LAP2\alpha^{-/-}$), showed infectivities of approximately 7.0% and 5.0% (lanes 7 and 8). The mouse macrophages were inherently more resistant to HIV-1 infection. Despite being infected with 5.2×10^5 infectious units (based on titration on NIH 3T3 cells) of virus per 1.0×10^5 cells, the infectivity on the mouse macrophages ranged between 4.5 and 7.0%.

Analysis of HIV-1 infection in the presence of dominant-interfering human emerin. The N-terminal LEM domain of emerin is necessary for interaction with BAF (14). Mutant emerin that fails to interact with BAF has been reported to impair HIV-1 infection (11). Because our prior analyses did not provide evidence for an essential role of emerin in retroviral infection of mouse cells, we sought to examine whether such a role was confined to human cells. HeLa cells stably expressing wild-type and LEM mutated forms of emerin were derived and tested for sensitivity to HIV-1 infection. However, no differences were observed in susceptibility relative to control lines (data not shown).

Because expression levels of mutant emerin might have been inadequate to interfere with infection in the stable cell lines, we reexamined the effect of LEM mutated forms of emerin via transient overexpression in HEK 293T cells. Two different LEM mutants were used: (i) the m24 LEM mutant characterized by Lee and colleagues as a mutant with four alanine substitution mutations in the LEM that is unable to bind BAF (14), and (ii) the ΔLEM mutant described by Jacque and Stevenson (11). Human 293T cells were transiently cotrans-

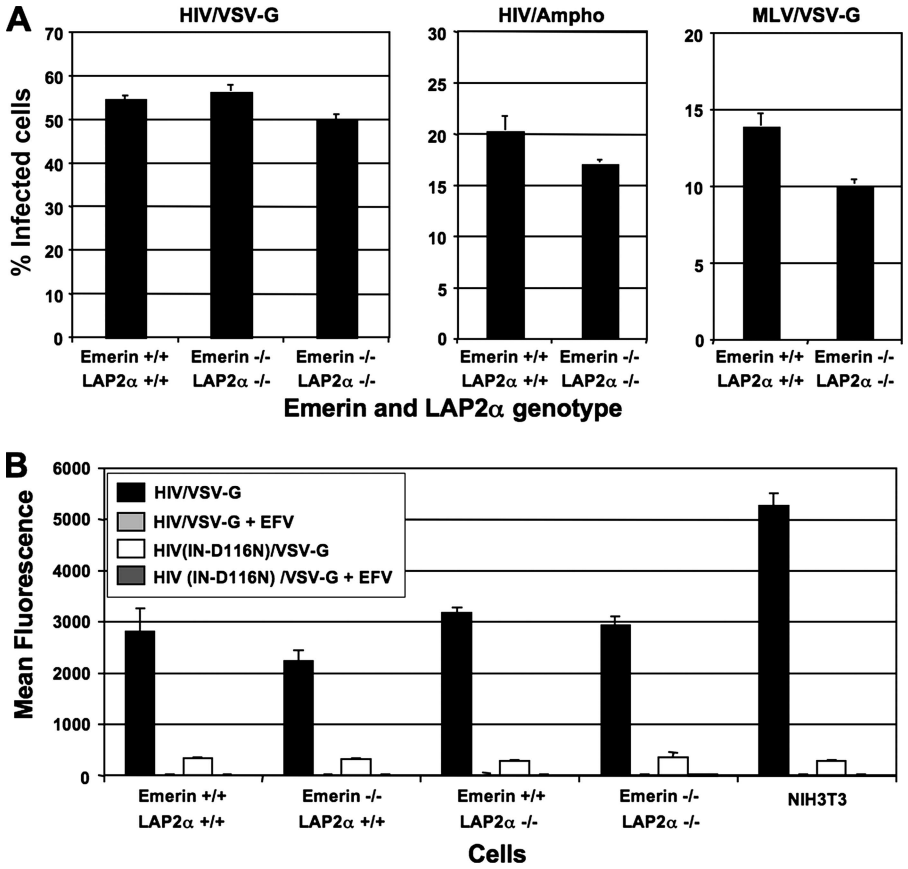

FIG. 3. HIV-1 and MLV can infect MEFs that are knocked out for genes encoding both emerin and LAP2α. (A) Infection efficiency of HIV/VSV-G, HIV/Ampho, or MLV/VSV-G was examined in emerin and LAP2α double knockout MEFs compared to wild-type littermate control MEFs. The percent infectivity represents the percentage of GFP-expressing MEFs 48 h postinfection with HIV-1 or MLV vectors. Each data set represents the mean of three separate wells of infected cells, and error bars indicate standard deviations. (B) The infectivity of HIV/VSV-G and $HIV^{IN\text{-}D116N}$/VSV-G was examined in wild-type, emerin knockout, LAP2α knockout, or emerin and LAP2α double knockout MEFs and NIH 3T3 cells. The cells were infected with an equal dose (200 ng p24 antigen) of virus. Infection was measured in the absence and presence of the nonnucleoside reverse transcriptase inhibitor EFV (100 nM). Infectivity was measured as the mean fluorescence intensity of GFP expression from infected cells to increase sensitivity for differences in gene expression. Each data set represents the mean of three separate wells of infected cells, and error bars indicate standard deviations.

fected with pDsRed-monomer-N1 and either empty vector, pEmerin(wild-type), pEmerin(m24-LEM), or pEmerin(ΔLEM) expression constructs. These transiently transfected cells were subsequently infected with HIV/VSV-G or MLV/VSV-G, and the percentage of cells expressing GFP was examined after 2 days. In individual samples, no differences between the infection rate of positively transfected cells (DsRed positive) and untransfected cells (DsRed negative) were observed (data not shown). Significantly, DsRed-positive cells expressing emerin with LEM mutations were as permissive to HIV-1 infection as cells expressing empty vector or high levels of wild-type emerin (Fig. 5A, left panel). Infection by MLV appeared to increase slightly in the presence of emerin mutants (Fig. 5A, right panel). A fraction of the transfected cells was lysed and analyzed by Western blotting for emerin levels (Fig. 5B). Exogenously expressed wild-type and mutant emerin were present in vast excess relative to endogenous emerin, such that endogenous protein in control samples was undetectable under the short exposure time needed to detect the transfected proteins (Fig. 5B, top panel). The blot was reprobed with monoclonal antibody to α-tubulin (Sigma) to confirm cell lysate loading consistency (bottom panel).

DISCUSSION

The ability of viruses to coopt host cell proteins and machinery is fundamental to their propagation and spread. Productive infection by retroviruses like HIV-1 and MLV requires integration of the virus into the host genome. The nuclear chromatin-associated protein BAF and nuclear lamina-associated LEM domain proteins emerin and LAP2α have been suggested to assist in targeting the viral PICs to chromatin during integration. Prior investigation of BAF demonstrated its presence in viral PICs and suggested its requirement for integration in vitro (6, 16, 17, 29). The association of emerin and LAP2α with PICs is thought to be indirect and via LEM domain interactions with BAF (11, 30). Functional evaluation of emerin and LAP2α in retroviral infection has relied heavily on RNAi experiments but has resulted in conflicting data regarding the role of these proteins in HIV-1 and MLV infection (11,

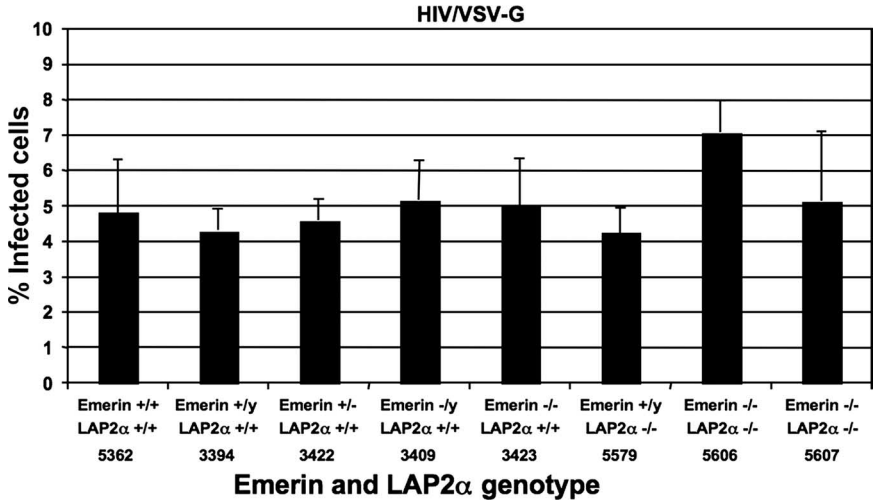

FIG. 4. HIV-1 can infect primary mouse macrophages from emerin and LAP2α knockout mice. Infection efficiency of HIV/VSV-G was examined in macrophages from emerin knockout, LAP2α knockout, and emerin and LAP2α double knockout mice compared to wild-type littermate controls. At 60 h postinfection, the cells were stained for the F4/80 macrophage-specific marker. The percent infectivity represents the percentage of GFP-positive cells in the F4/80-positive population. Each data set represents the mean of four separate wells of infected cells, and error bars indicate standard deviations.

27, 30). While MEFs from emerin-deficient mice had also been previously tested (27), this analysis was limited to one knockout line and did not include emerin-deficient macrophages from adult mice. Moreover, LAP2α knockout mice were only recently derived, allowing investigation of retroviral permissivity in MEFs or macrophages singly and doubly ablated for LEM domain proteins. With these tools, we demonstrate that both HIV-1 and MLV can infect dividing MEFs and that HIV can infect nondividing mouse macrophages from emerin knockout, LAP2α knockout, or emerin and LAP2α double knockout mice as efficiently as cells from wild-type littermate mice. Moreover, human cell lines expressing wild-type or dominant-negative mutant emerin were found to be equally susceptible to HIV-1. Based on these findings, we conclude that these two LEM domain-containing proteins are not required for infection of mouse cells by HIV-1 and MLV.

Because Jacque and Stevenson (11) conducted experiments in human cells, it could be argued that cross-species differences between mouse and human emerin or LAP2α orthologs prevent productive interaction with HIV-1 and MLV PICs within the murine cells. However, at least with regards to MLV infection, murine cells are the most relevant targets. Thus, because emerin or LAP2α removal from mouse cells has no clear effect on MLV vector transduction, these proteins are probably irrelevant to this process. Prior analysis in mouse cells by others appeared to demonstrate that MLV replication was impaired after depletion of LAP2α by RNAi, but these effects manifested only after cycles of replication, and it was not determined if they occurred early in the viral life cycle (30).

The differences observed in the essential nature of emerin or LAP2α for HIV-1 infection by Shun and colleagues (27) and what we have presented here are more difficult to reconcile with the findings of Jacque and Stevenson (11). In our work, we have primarily relied on mouse knockout cells to avoid the variability that is sometimes observed when using RNAi-based assays. In prior studies, we observed HIV-1 vector transduction to be comparable in mouse and human fibroblasts, indicating efficiency in early replication steps (2). It thus seems unlikely that HIV-1 utilizes entirely different pathways when infecting mouse versus human cells, as this would suggest that contacts with putative cofactors are simply incidental. Indeed, essential cofactors such as LEDGF/p75 are required by HIV-1 both in mouse and human cell types (19, 28, 33). Interestingly, analysis of LEDGF/p75 indicates that it may direct HIV-1 PICs to the host cell chromatin after nuclear entry, a function that would seemingly be redundant to the proposed role of emerin in HIV-1 infection (10, 20, 21, 31, 34).

Although beyond the scope of our analyses, it is possible that our assay systems fail to reveal subtle or cell-specific phenotypes in the absence of emerin or LAP2α. For example, integration site specificity could be altered in cells lacking either factor without impairing infection efficiency. Alternatively, MLV or HIV-1 infection of specific cell types or under different cell growth conditions may elicit a greater requirement for these factors. However, even with these limitations, retroviral infection efficiency in highly permissive or less permissive primary cell types, MEFs and macrophages, respectively, is strikingly consistent in the presence or absence of the LEM domain proteins. In addition, the emerin and LAP2α deficiencies did not appear to change the biology of HIV-1 infection by permitting accumulation of vDNA in the absence of integration. Our data indicate that HIV-1 IN function was required to establish infection in the absence of either or both LEM domain proteins.

Analysis of protein functional relevance in cells from gene-ablated mice can present other concerns. During the development of cell lines from the knockout animals, it is conceivable that there could be some compensatory upregulation of other nuclear envelope proteins, which would restore HIV/MLV infectivity to levels observed in wild-type cells. Because both emerin and LAP2α had been proposed to play similar roles in retroviral infection, our experiments with double knockout

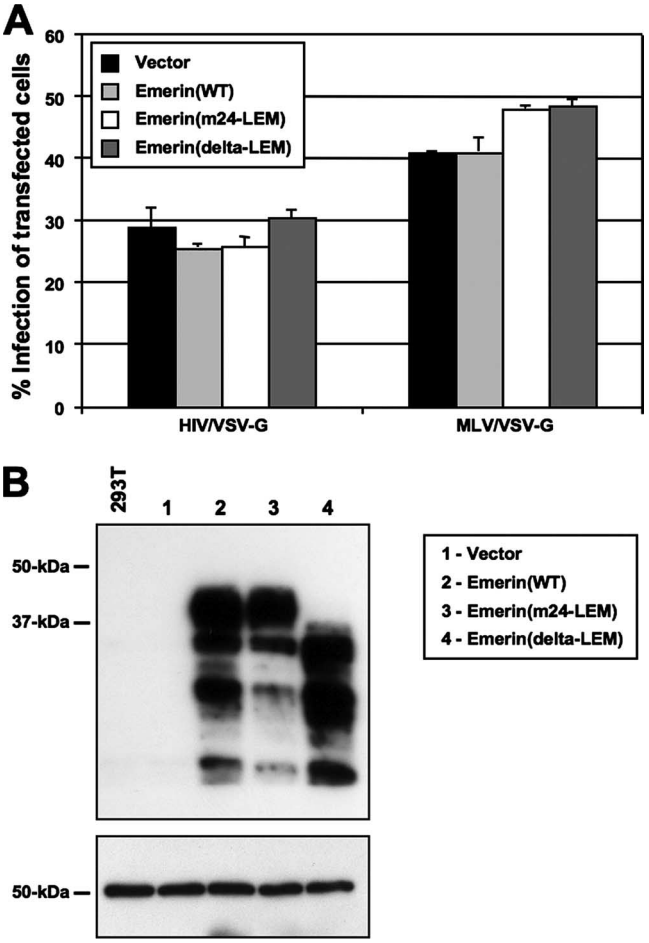

FIG. 5. HIV-1 infection in cells expressing wild-type and dominant-negative emerin. (A) 293T cells transiently transfected with pIRES-puro3, pEmerin(WT)-IRES-puro, pEmerin(m24-LEM)-IRES-puro, or pEmerin(delta-LEM)-IRES-puro were infected with HIV/VSV-G or MLV/VSV-G at similar efficiency. Cells were cotransfected with pDsRed-monomer-N1 (Clontech) at a threefold-lower molar amount as a transfection control. The percent infectivity was measured as the percentage of GFP-positive cells in the DsRed-positive population as measured by FACS. (B) Top panel: expression of wild-type emerin or mutant emerin proteins in 293T cells used in the above infections was checked using Western blotting by probing with an anti-emerin polyclonal antiserum (Santa Cruz Biotechnology, Inc.). Bottom panel: the blot was reprobed with monoclonal antibody to α-tubulin (Sigma) to confirm cell lysate loading consistency.

cells ruled out the possibility that these proteins were functionally redundant, thus masking effects in single knockout cells. While expression of other LEM domain-containing proteins is not significantly increased in emerin or LAP2α knockout lines (data not shown), another LEM domain protein could, in theory, productively interact with PICs in the absence of competing factors. Nonetheless, even if a third factor supported retroviral infection and/or enhanced BAF interactions with chromatin in the double knockout cells, it would again imply that emerin and LAP2α are not essential for HIV/MLV infection and are easily replaced by other proteins.

Despite caveats, an advantage of using knockout cells is the complete gene-level ablation of the factor being characterized. While RNAi-based techniques permit the rapid analysis of gene function in a variety of cell types, it is difficult to deplete all the mRNA for a targeted factor. It is also conceivable that a rapid depletion of emerin or LAP2α by RNAi could have collateral effects on BAF stability and/or distribution that contribute to the reduced permissivity of these cells. Because of the clear discordance in RNAi-dependent analyses of the role of emerin and LAP2α in retroviral infection in preceding studies, we also employed a third strategy to investigate emerin function in human cells by expressing mutant forms of emerin previously argued to have dominant-negative effects on HIV-1 infection (11). However, using two different LEM domain mutated forms of emerin, we could not reproduce an inhibition of HIV-1 infection even in the presence of extremely high levels of mutant proteins. These results were consistent with our observations using knockout mouse cells and further undercut an essential role for emerin in human cells.

Interestingly, Shun and colleagues have also failed to detect an effect on retroviral infection after small interfering RNA-dependent depletion of BAF in human target cells (27). An essential role for BAF in retroviral infection in vivo is integral to models that consider the potential influence of LEM domain proteins. BAF is proposed to act as the linchpin that connects these proteins to the retroviral PIC. Future studies with BAF-deficient mouse cells may be revealing in this regard. Recent studies with poxviruses suggest that BAF may, in some circumstances, act as an antiviral factor (37). Further examination of the role of BAF during retroviral infection, both alone and together with other nuclear lamin proteins, will be of great interest given the clear interaction of BAF with retroviral PICs.

ACKNOWLEDGMENTS

We thank Zandrea Ambrose and Derya Unutmaz for reagents and advice and Michael Emerman, Alan Engelman, and Mario Stevenson for discussions.

This research was supported by the Intramural Research Program of the National Cancer Institute.

The content of this publication does not necessarily reflect the views or policies of the Department of Health and Human Services, nor does mention of trade names, commercial products, or organizations imply endorsement by the U.S. Government.

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