JOURNAL OF VIROLOGY, May 2008, p. 4665–4670 Vol. 82, No. 9
0022-538X/08/$08.00+0 doi:10.1128/JVI.02403-07
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The Ezrin-Radixin-Moesin Family Member Ezrin Regulates Stable Microtubule Formation and Retroviral Infection▽

Juliane Haedicke,[1] Kenia de los Santos,[2] Stephen P. Goff,[2] and Mojgan H. Naghavi[1,2]*

Centre for Research in Infectious Diseases, School of Medicine and Medical Science, University College Dublin, Belfield, Dublin 4, Ireland,[1] and Department of Biochemistry and Molecular Biophysics, Howard Hughes Medical Institute, Columbia University, New York, New York 10032[2]

Received 7 November 2007/Accepted 19 February 2008

We recently identified the cytoskeletal regulatory protein moesin as a novel gene that inhibits retroviral replication prior to reverse transcription by downregulation of stable microtubule formation. Here, we provide evidence that overexpression of ezrin, another closely related ezrin-radixin-moesin (ERM) family member, also blocks replication of both murine leukemia viruses and human immunodeficiency virus type 1 (HIV-1) in Rat2 fibroblasts before reverse transcription, while knockdown of endogenous ezrin increases the susceptibility of human cells to HIV-1 infection. Together, these results suggest that ERM proteins may be important determinants of retrovirus susceptibility through negative regulation of stable microtubule networks.

Recently, a number of novel antiviral mechanisms have come to light. It has been suggested that these pathways should be differentiated from classical innate immunity by the designation "intrinsic" immunity (3, 19). Many owe their discovery to studies of retrovirus biology, including Fv4 (13), TRIM5α (30), the APOBECs (29), Fv1 (1), and ZAP (11); some of these also affect the replication of other viruses (2, 8, 24, 34).

In addition to these restriction factors, whose primary function appears to be to prevent retrovirus replication, an increasing number of genes that can modulate cell sensitivity to retrovirus infection in a more indirect way are also being identified. These are exemplified by Murr1, which inhibits human immunodeficiency virus type 1 (HIV-1) growth in unstimulated $CD4^+$ T cells (9); protein arginine methyltransferase PRMT6, whose knockdown increases the susceptibility of HIV-1-resistant HEK293T cells to infection (4); fasciculation and elongation protein zeta 1 (FEZ-1), whose overexpression blocks the transport of retroviral DNA into the nucleus (22); and p21, which affects the sensitivity of HIV-1-resistant hematopoietic stem cells to infection (38, 39). The growing list of host proteins suggests that more such factors are yet to be identified. Understanding how these factors modulate virus replication will provide invaluable insights into host-virus interactions and could lead to the development of novel antiviral treatment strategies.

We recently demonstrated that moesin, a member of the closely related cytoskeletal regulatory family of ezrin-radixin-moesin (ERM) proteins, regulates stable microtubule formation and limits retroviral infection (23). ERM proteins display degrees of redundancy in certain functions (5, 14). However, it is unknown if all ERM family members confer resistance to viral infection. In this study, we examined another family member, ezrin, for similar activity. To test whether ezrin could affect retroviral replication, full-length rat ezrin was amplified and subcloned into the mammalian expression vector pcDNA3.1$^+$ (Invitrogen) using the following primers: forward, Ezr-S1-EcoRI, 5′-GCTGATGAATTCGCCACCATGCCCAAGCCAATCAACGTCC-3′; reverse, Ezr-A1-NotI, 5′-GCAACTGCGGCCGCCTACATGGCCTCAAACTCGTCGATGCG-3′. Rat2 cells (3×10^6) were then transfected with 4 μg of the ezrin construct or control empty pcDNA3.1$^+$ vector and selected in G418 (500 μg/ml) 48 h posttransfection. Several resistant clones of each stable line were isolated, and levels of ezrin expression were determined by quantitative real-time PCR (QPCR) measurement of mRNAs (Fig. 1A) using Sybr green JumpStart *Taq* ReadyMix (Sigma) (22). Transgene ezrin transcript levels were determined using one primer specific to ezrin sequences and one primer unique to the vector sequences (EZR-A3, 5′-CTCTGAGGGATAAGTCTCTC-3′, and pCMV-forward, 5′-CGCAAATGGGCGGTAGGCGTG-3′, respectively). The number of target copies in each sample was interpolated from its detection threshold (C_T) value using a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) plasmid standard curve (22). As expected, no transgene expression of ezrin was detected in the wild-type (wt) Rat2, the empty pcDNA:9 vector, or the previously described control virus mutant R4-7 line (10) (Fig. 1A).

These clones were then tested in transduction assays using Moloney murine leukemia virus (MoMLV)-puro pseudotyped with vesicular stomatitis virus glycoprotein as described previously (22). All four tested variants of ezrin conferred resistance to infection relative to control Rat2 or empty vector lines (Fig. 1B). Although the degree of resistance did not directly correlate with ezrin expression levels, this likely reflects the clonal nature of the lines generated. To examine whether the resistance induced by ezrin was virus specific and/or envelope dependent, two of these lines were also tested for susceptibility to HIV-1-puro pseudotyped with amphotropic envelope protein (HIV-1-ampho-puro) (Fig. 1C). Again, these lines significantly inhibited the replication of HIV-1 vectors, which enter the cell by fusion at the plasma membrane, indicating a block in a general aspect of the retroviral life cycle independent of the route of entry. As expected, high levels of resistance to both

* Corresponding author. Mailing address: Centre for Research in Infectious Diseases, University College Dublin, Belfield, Dublin 4, Ireland. Phone: 353 1 716 1223. Fax: 353 1 716 1239. E-mail: mojgan.naghavi@ucd.ie.
▽ Published ahead of print on 27 February 2008.

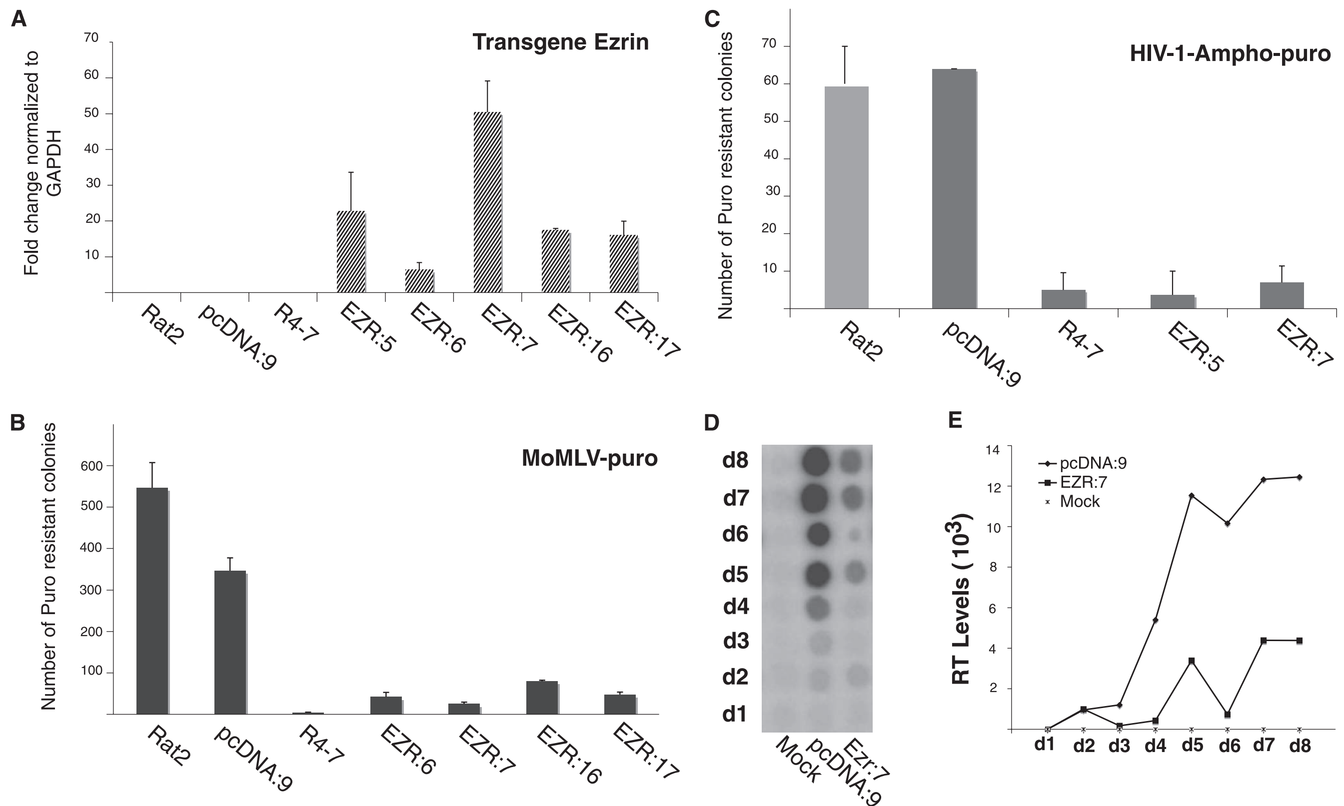

FIG. 1. Expression of ezrin confers resistance to retroviral infection. (A) QPCR showing the level of ezrin expression in Rat2 cells. Cytoplasmic RNA prepared from wt Rat2 fibroblasts (Rat2), control virus-resistant cells (R4-7), Rat2 cells stably transduced with the empty vector (pcDNA:9), and Rat2 cells stably expressing ezrin (EZR:5, EZR:6, EZR:7, EZR:16, and EZR:17) was converted to double-stranded cDNA and used as template for QPCR. The levels of transgene ezrin transcripts were measured using one primer unique to vector sequences. The change ratio (*n*-fold) between the Rat2 mutant lines and the control empty vector pcDNA:9 line are median copy numbers normalized to GAPDH copy numbers obtained in triplicate from two independent RNA preparations. (B and C) Overexpression of ezrin in Rat2 cells blocks retrovirus infection. The same lines as in panel A were incubated with various amounts of MoMLV-puro (B) or HIV-ampho-puro (C). Cells were selected in medium containing puromycin, and the number of transduced colonies was counted after 8 to 12 days. Similar results were obtained in at least three independent experiments. (D) Replication of MoMLV in a spreading infection measured by reverse transcriptase (RT) assay. Spots indicate the amount of labeled nucleotide incorporated in an exogenous RT assay on a homopolymer template by virion-associated RT. Equal amounts of virus were used to infect Rat2 cells stably transduced with the empty vector (pcDNA:9) or stably expressing ezrin (EZR:7). Culture supernatants were collected on successive days as indicated, and virus production was monitored by RT assay. Mock, no virus was applied. (E) Quantification of viral replication assay results shown in panel C. Dot intensities were normalized against uninfected cell samples.

MoMLV-puro (Fig. 1B) and HIV-1-puro (Fig. 1C) challenges were also observed for the previously described virus-resistant rat fibroblast line R4-7, which was included as a positive control (10). Ezrin-overexpressing lines also inhibit replication of wt MoMLV virus, as measured by the appearance of reverse transcriptase in culture media of cells infected with 10-fold serial dilutions of MoMLV (22) (Fig. 1D and E). In conclusion, ezrin overexpression induces a potent resistance to infection by both genetically marked and wt retroviruses.

To determine the point in the viral life cycle at which the block to infection occurs, viral DNA synthesis was examined after infection of one ezrin-overexpressing line with ecotropic MLV-green fluorescent protein (GFP) (10). At 24 h postinfection, Hirt DNA was isolated and used as template for quantitative PCR (22). Primers to amplify GFP sequence, the MLV minus-strand strong-stop DNA, the plus-strand viral DNA, and the MLV long terminal repeat (LTR)-LTR junction were previously reported (22). Very low levels of linear viral DNA (Fig. 2A), minus-strand strong-stop DNA (Fig. 2B) and plus-strand DNA (Fig. 2C) were produced in EZR:6 cells overexpressing ezrin, suggesting that the block occurs before the initiation of reverse transcription.

ERM proteins colocalize with actin filaments at cell surface structures, cross-link actin in the cortical layer, and control cell shape and movement (33). They have been suggested to be important in establishing leading plasma membranes and directional cell migration (25, 28). Several studies suggest that, upon entry, retroviruses use actin filaments for short-range transport in the cell periphery, while they seem to hijack microtubule motor dyneins for long-range transport from the cell periphery to the nucleus (21). Disruption of the actin cytoskeleton using cytochalasin D (Sigma) reduced viral infectivity in both control pcDNA:9 and ezrin-overexpressing EZR:6 lines (Fig. 3A). Combined with previous reports that cortical actin facilitates trafficking of viral cores at the cell periphery after entry (6, 16, 17, 20, 32), this result suggests that the effects of ezrin occur after initial actin-mediated entry events and that moesin and ezrin may function in the subsequent transition of

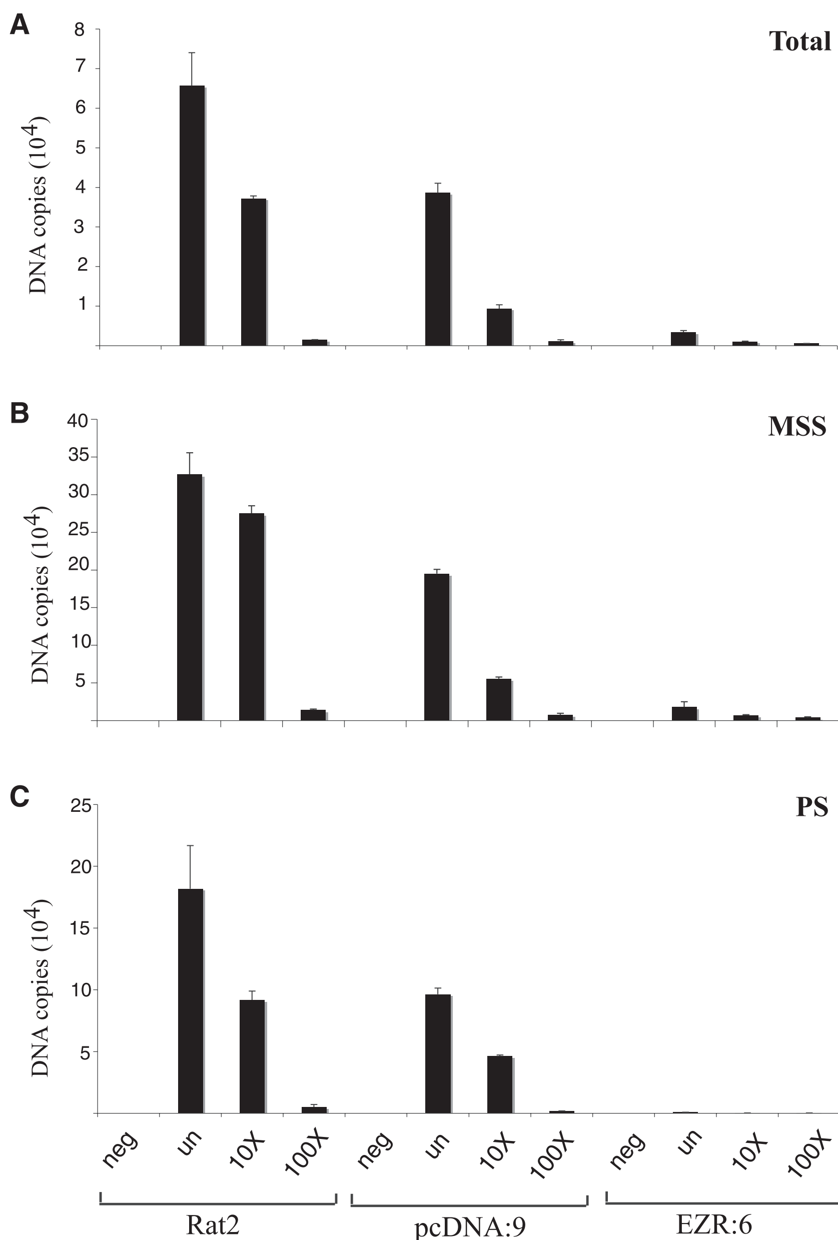

FIG. 2. Analysis of the block to virus infection using quantitative PCR. wt Rat2 cells, the control empty vector pcDNA:9 line, and one of the ezrin-overexpressing lines (EZR:6) were infected with either undiluted (un) or 10-fold serially diluted (10×, 100×) ecotropic MLV-GFP. Uninfected cells were included as a negative control (neg) for each sample. Total DNA was isolated at 24 h after infection, and the amount of viral DNA synthesized in the infected cells was measured by quantitative PCR. By using primers specific to GFP sequences, minus-strand strong-stop DNA, or plus-strand DNA, the amounts of total linear viral DNA (Total) (A), minus-strand strong-stop DNA (MSS) (B), or plus-strand DNA (PS) (C) were determined, respectively. Each DNA sample was assayed in duplicate at a minimum of three different time points.

viral cores to or along the microtubule network, allowing reverse transcription and movement toward the nuclear periphery. Our previous findings suggested that moesin disrupts a small subset of detyrosinated (stable) microtubules (18), termed Glu-MTs, involved in vesicle and cytoskeletal trafficking (37), which could affect the transition and/or motility of viral cores (23). To test if ezrin also specifically affected stable microtubule formation, we examined the levels of Glu-MTs and tyrosinated tubulin (termed Tyr-MTs) in ezrin-overexpressing and control empty vector lines. Cells grown on coverslips were stained for stable and dynamic MTs using rabbit polyclonal (SG) or rat monoclonal (YL1/2) antibodies, respectively, and detected using preabsorbed fluorescence-labeled secondary antibodies (Jackson ImmunoResearch Laboratories) and an Olympus BX50 microscope (23). Ezrin-overexpressing lines EZR:6 (Fig. 3B) and EZR:7 (data not shown) showed significantly lower levels of Glu-MTs (44% and 39% of cells staining positive, respectively) than did control empty pcDNA:9 cells (68% positive) (Fig. 3B). The effect was specific for stable microtubules, as tyrosinated microtubule staining

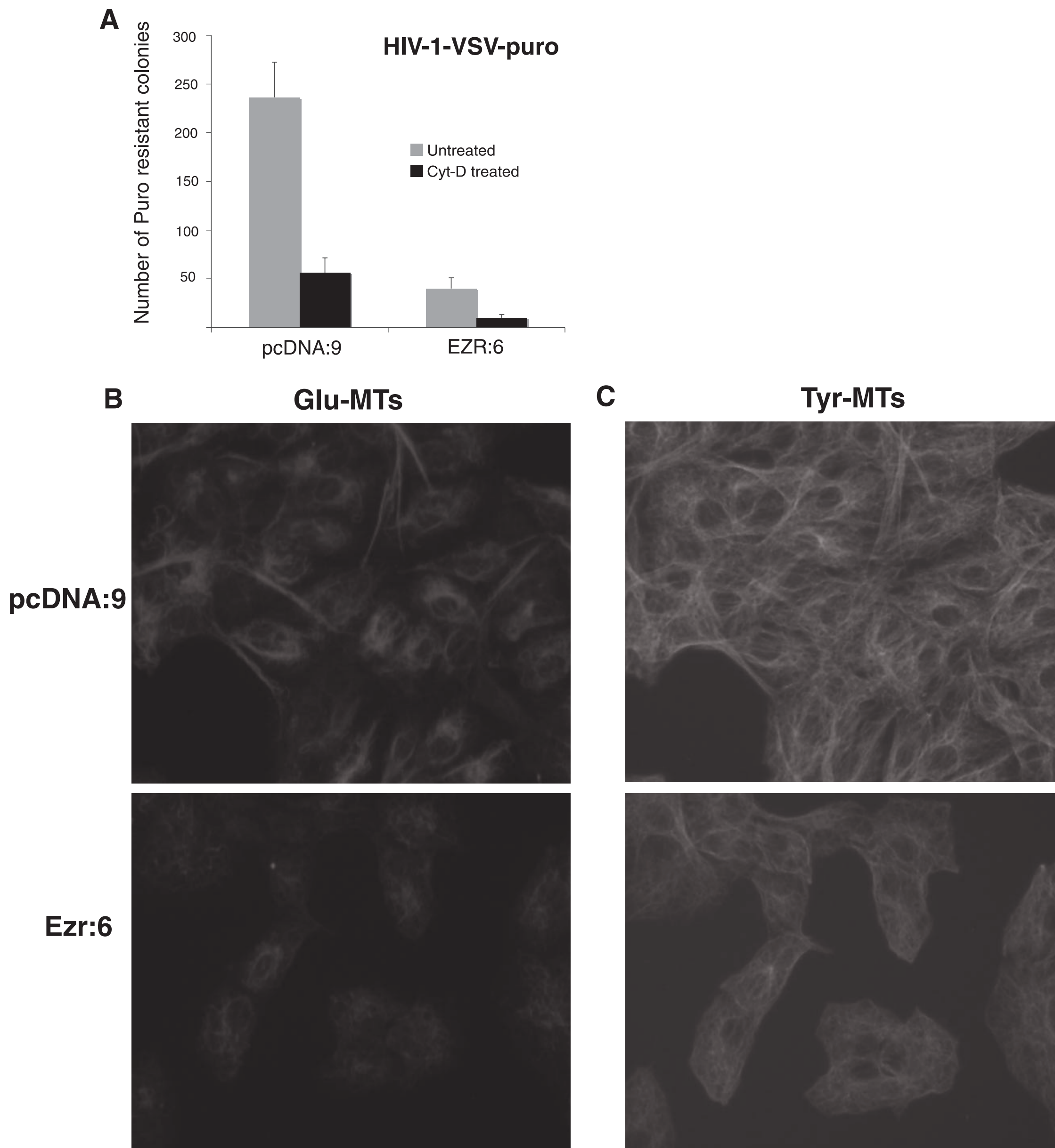

FIG. 3. The role of cytoskeletal networks during viral infection of ezrin-overexpressing lines. (A) The empty vector pcDNA:9 line and the ezrin-overexpressing EZR:6 cells were incubated in the presence or the absence of 0.4 μg/ml cytochalasin D (Cyt-D) for 5 h. The cells were then infected with various amounts of HIV-1-puro carrying a vesicular stomatitis virus (VSV) glycoprotein envelope protein in the presence or the absence of Cyt-D, and the infection assay was carried out as described in the legend to Fig. 1. Similar results were obtained in at least three independent experiments. (B and C) Ezrin overexpression specifically affected stable microtubule formation. The same cell lines were fixed and stained with a rabbit polyclonal antibody for stable Glu-MTs (B) and a rat monoclonal antibody for dynamic Tyr-MTs (C), followed by incubation with fluorescently labeled secondary antibodies. Images were taken as described in the text.

was similar to control pcDNA:9 cells (Fig. 3C). Furthermore, ezrin overexpression did not affect other parameters, such as cell shape, size, or doubling time (data not shown). Overall, the inhibitory effects of actin-depolymerizing agents on viral infection even in ezrin-overexpressing cells and the inhibitory effects of ezrin on viruses that enter the cell by either fusion or endocytosis suggest that ezrin blocks infection postentry, after transition from the actin cytoskeleton. The failure to detect strong-stop DNA suggests that ezrin's effects on the microtubule network of the cell likely block events that lead to reverse transcription complex/preinitiation complex formation or transport, effecting an early block to infection.

The endogenous levels of the related ERM proteins ezrin and moesin in both Rat2 and the human rhabdomyosarcoma TE cells were determined by QPCR measurement of mRNAs (Fig. 4A and B) using primers specific to rat (23) or human ezrin and moesin sequences (hEzr-S, 5′-CCAGGCCCGAGATGAGAATA-3′; hEzr-A, 5′-TGCCACGTTTCCTTTAATGATG-3′; and hMsn-S2, 5′-GCCAATGACATGATCCATGC-3′; hMsn-A2, 5′-CCTAGGTCCTGTCTCATTCC-3′, respectively)

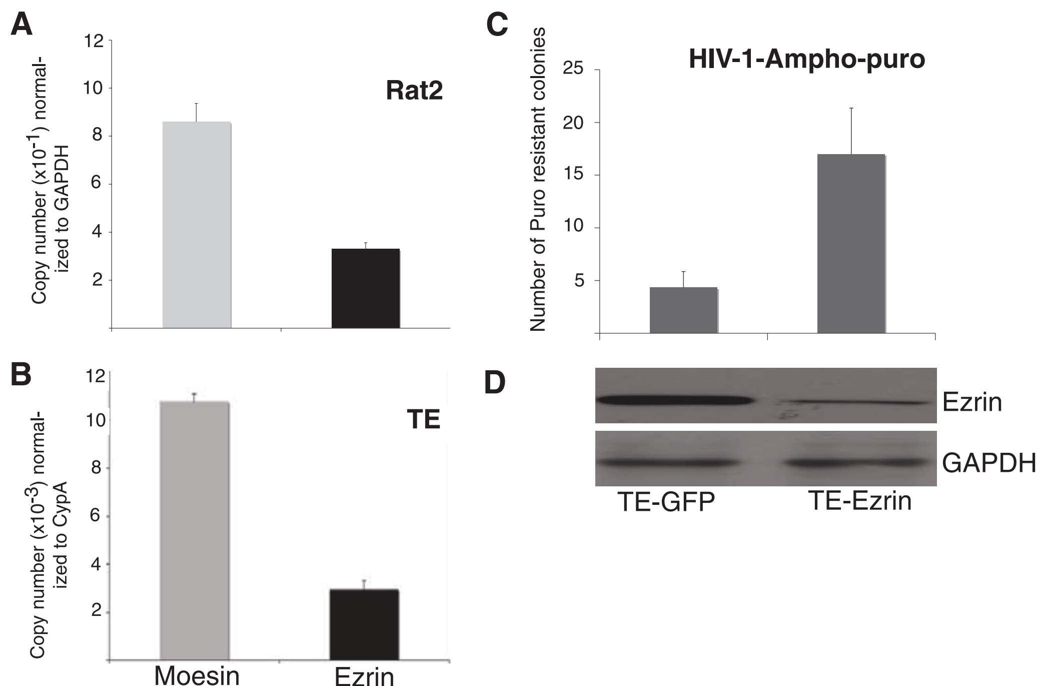

FIG. 4. RNA interference to ezrin increases the virus susceptibility of human TE cells. (A and B) QPCR showing the endogenous levels of ezrin and moesin in Rat2 and TE cells, respectively. Cytoplasmic RNA was converted to double-stranded cDNA and used as template for quantitative PCR. Specific primers were used to measure the levels of endogenous ezrin and moesin transcripts. Median copy numbers for each transcript were normalized to GAPDH (Fig. 4A) or cyclophilin A (CypA) (Fig. 4B) copy numbers obtained in triplicate from two independent RNA preparations. (C) TE cells were transfected with either a nonspecific GFP duplex or a pool of three independent siRNAs targeting human ezrin on 2 consecutive days with equal amounts of RNA duplexes and subsequently seeded and infected with various amounts of HIV-1-ampho-puro as described in the legend to Fig. 1C. Similar results were obtained in at least three independent experiments. (D) Western blotting showing the level of ezrin knockdown. Upper panel, endogenous ezrin expression was detected in the same lines using antiezrin antibodies. Lower panel, loading of equal amounts of protein was confirmed using GAPDH antibodies.

as described above. The number of target copies in each sample was interpolated from its C_T value using a cyclophilin A plasmid (a kind gift from J. Luban, Switzerland Institute for Research in Biomedicine, Bellinzona, Switzerland) standard curve.

To test whether endogenous levels of human ezrin influence virus susceptibility, RNA interference was used to reduce ezrin expression in TE cells, and cells were challenged with genetically marked retroviruses. TE cells were transfected with 300 pmol of either a pool of three short interfering RNAs (siRNAs) (Santa Cruz) specific to human ezrin or a nonspecific control siRNA duplex targeted to GFP on 2 consecutive days. Cells were subsequently seeded, infected with various amounts of HIV-1-puro, and selected (22). Ezrin-specific RNA interference duplexes induced a fourfold increase in the susceptibility of these cells to HIV-1-puro infection, while the nonspecific siRNA duplex had no effect (Fig. 4C). Levels of ezrin expression were measured by Western blotting of whole-cell lysates fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and probed with the indicated antiserum: anti-ezrin (a gift from A. Bretscher, Cornell University, Ithaca, NY) or anti-GAPDH (Santa Cruz). The partial increase (3.9-fold) in susceptibility correlated with decreased ezrin expression (3.8-fold or 60% reduction in the intensity of the band) (Fig. 4D, upper panel), suggesting that even endogenous levels of ezrin are sufficient to limit virus infection.

The fact that ezrin and moesin confer resistance to retroviral infection suggests that a common mechanism and protein domains or interacting proteins exist among this family of cytoskeletal regulators that block retroviral replication. This may occur indirectly through their effect on stable MT formation and the factors involved therein to prevent viral movement within the cell. Stable microtubule formation is influenced by cytoskeletal regulatory Rho-GTPases (7, 27), which are regulated by ERM proteins (14, 31, 35). ERM protein overexpression may therefore sequester a cellular protein(s) involved in Rho-GTPase pathways that in turn regulate stable microtubules. Alternatively, ERM proteins or ERM-associated factors may directly interact with viral factors, as several studies have shown the presence of both ezrin and moesin inside HIV-1 virions (26), the binding of ERM proteins to the envelope protein gp120 (12), and a correlation between ERM/ERM-related proteins and HIV-1 pathogenesis (15, 36). The ERM proteins moesin and ezrin are clearly important natural determinants of cellular sensitivity to retroviral infection. An understanding of whether they play a direct role in viral replication or inhibit replication through their effect on stable MT formation will provide important insights into early events in the retroviral life cycle and the cortical actin-microtubule transition.

We thank A. Bretscher for kindly providing us with the ezrin antibodies.

M.H.N. is supported by the Science Foundation Ireland (SFI) under grant no. 06/IN.1/B78, and S.P.G. was supported by a grant from the

NCI (R37-CA30488) and is an Investigator of the Howard Hughes Medical Institute.

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