Visna Virus-Induced Activation of MAPK Is Required for Virus Replication and Correlates with Virus-Induced Neuropathology

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It is well accepted that viruses require access to specific intracellular environments in order to proliferate or, minimally, to secure future proliferative potential as latent reservoirs. Hence, identification of essential virus-cell interactions should both refine current models of virus replication and proffer alternative targets for therapeutic intervention. In the present study, we examined the activation states of mitogen-activated protein kinases (MAPKs), ERK-1/2, in primary cells susceptible to visna virus and report that virus infection induces and sustains activation of the ERK/MAPK pathway. Treatment of infected cells with PD98059, a specific inhibitor of the ERK/MAPK pathway, abolishes visna virus replication, as evidenced by extremely low levels of Gag protein expression and reverse transcriptase activity in culture supernatants. In addition, although visna virus-induced activation of MAPK is detectable within 15 min, early events of viral replication (i.e., reverse transcription, integration, and transcription) are largely unaffected by PD98059. Interestingly, further examination demonstrated that treatment with PD98059 results in decreased cytoplasmic expression of *gag* and *env*, but not *rev*, mRNA, highly suggestive of an ERK/MAPK-dependent defect in Rev function. In vivo analysis of ERK-1/2 activation in brains derived from visna virus-infected sheep demonstrates a strong correlation between ERK/MAPK activation and virus-associated encephalitis. Moreover, double-labeling experiments revealed that activation of MAPK occurs not only in cells classically infected by visna virus (i.e., macrophages and microglia), but also in astrocytes, cells not considered to be major targets of visna virus replication, suggesting that activation of the ERK/MAPK pathway may contribute to the virus-induced processes leading to neurodegenerative pathology.

Historically, visna virus and caprine encephalitis virus are prototypes of the lentivirus subfamily of retroviruses, which also includes equine infectious anemia virus, bovine immunodeficiency virus, feline immunodeficiency virus, simian immunodeficiency virus, and human immunodeficiency virus (HIV). Visna virus causes interstitial pneumonitis, arthritis, mastitis, and inflammatory infiltration of the central nervous system (CNS) in sheep, months to years after initial infection, typifying the slow progressive onset of disease, hallmark of lentivirus infection (77, 78, 91). In vivo, the predominant target cells for visna virus infection are of the monocyte/macrophage lineage; however, virus replication is tightly regulated by both viral and cellular factors, such that infected monocytes do not express significant levels of viral mRNA or protein prior to maturation and differentiation into macrophages (32, 33, 58, 77, 78, 91).

Differentiation of monocytes into macrophages is accompanied by increased production of Fos and Jun, as well as enhanced AP-1-mediated transcription (15, 31, 46, 76). Both of these features provide a more amenable environment for visna virus replication at least in part, because optimal viral transcription requires an AP-1 site proximal to the TATA sequence in the viral long terminal repeat (14, 39). Moreover, the visna virus transactivating protein Tat has been shown to interact directly with Fos and Jun, as well as the TATA binding protein (54). The exact mechanism of transactivation has yet to

be defined, but the present model depicts targeting of Tat via an alpha-helical domain to the basic regions of Fos/Jun (or possibly Jun/Jun) dimers complexed to the AP-1 site in the viral long terminal repeat (11, 54). In this configuration, the activation domain of Tat associates with TATA binding protein, resulting in enhanced transcription initiation from the visna virus promoter.

The experiments described in this report were designed to assess the potential role of mitogen-activated protein kinases (MAPKs), specifically the extracellular regulated kinases, ERK-1/2, in visna virus replication. We chose to examine these MAPKs because ERK-1/2 have been implicated in the differentiation of monocytes to macrophages, induction of Fos and Jun, and activation of AP-1-dependent transcription (6, 22, 29, 41, 44, 65, 86). Like all MAPK pathways, the ERK-1/2 signaling pathway is comprised of exquisitely specific, sequentially activated protein kinases, the activities of which are subject to tight regulation (reviewed in references 12 and 16). Activation of ERK-1/2 is typically transient and occurs in response to numerous stimuli, including growth factors, cytokines, bacterial products, and cell-cell or cell-matrix interactions. Downstream substrates of ERK-1/2 include a wide variety of proteins, such as transcription factors, protein kinases, cytoskeleton-associated proteins, and phospholipases, illustrating how activation of ERK-1/2 can amplify the signal generated from a single stimulus into a multifaceted cellular response. Activation of ERK-1/2 requires coordinate phosphorylation of threonine and tyrosine residues contained within a characteristic TEY motif, a process carried out by the dual-specific MAPK/ERK kinases, MEK-1/2 (73). To date, ERK-1/2 are the

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only known substrates for MEK-1/2, which are inhibited specifically by PD98059 (2, 23).

We report that infection with visna virus *in vitro* induces rapid and sustained activation of the ERK-1/2 pathway. Inhibition of virus-induced MAPK activation using PD98059 is paralleled by a dose-dependent reduction of Gag protein expression and reverse transcriptase (RT) activity in culture supernatants. Treatment with PD98059 has no apparent effect on proviral DNA synthesis and therefore does not interfere with early steps of the replication process. However, treatment with PD98059 leads to a marked decrease in the cytosolic expression of *gag* and *env* mRNA, but not *rev* mRNA. These results implicate no profound defects in virus integration or transcription but rather suggest that activation of ERK-1/2 may be required for optimal Rev function. *In vivo* analysis of MAPK activation in brains derived from visna virus-infected sheep demonstrates a strong correlation between MAPK activation and visna virus-associated encephalitis, classically characterized by intense periventricular and perivascular infiltrates of macrophages and lymphocytes, multifocal demyelination, and astrocytic hyperplasia (89). Double-labeling experiments demonstrate that, in addition to macrophages and microglia, the predominant CNS cell types infected by visna virus *in vivo*, astrocytes also express activated MAPK. These results suggest that activation of MAPK correlates with visna virus-induced encephalitis and occurs in cells not considered to be major targets of virus replication but may nonetheless contribute to the virus-induced neurodegenerative disease processes in the CNS.

MATERIALS AND METHODS

Cell culture and reagents. Sheep choroid plexus (SCP) cells and goat synovial membrane (GSM) cells were obtained as previously described (56–58) and cultured in minimum essential medium with Earle's salts (EMEM) (Life Technologies, Gaithersburg, Md.) supplemented with 50- μ g/ml gentamicin (Life Technologies) and 10% fetal bovine serum (FBS) (Atlanta Biologicals, Norcross, Ga.). These primary cells represent the prototypic organ-specific target cells for *in vitro* studies of visna virus (89). Both cells are readily infectible and are found in the brain (SCP) and joints (GSM), the primary tissues in which visna virus-induced disease is manifested *in vivo*. Primary sheep macrophages were prepared as described previously (92). EMEM without methionine was obtained from Sigma (St. Louis, Mo.). PD98059 (Calbiochem; La Jolla, Calif.) and U0126 (Promega, Madison, Wis.) were diluted in dimethyl sulfoxide (DMSO) or methanol to generate working stocks of 5 and 10 mM, respectively. Neither inhibitor affected cell viability (assessed by trypan blue exclusion), steady-state levels of MAPK, or synthesis of GAPDH mRNA at the concentrations used in all experiments.

Virus infections. Visna virus strain 1514 has been described previously (18, 57, 64). SCP and GSM cells cultured to approximately 80% confluence were washed twice with serum-free EMEM and infected for ~16 h (37°C; 5% CO₂) with between 1×10^4 50% tissue culture infective doses (TCID₅₀) and 5×10^4 TCID₅₀ of visna virus 1514 in EMEM supplemented with 0.5% lamb serum, after which the virus was removed and replaced with EMEM supplemented with 0.5% lamb serum for the course of the experiment. For infections treated with PD98059, cells were washed twice with serum-free EMEM and incubated for the indicated times with PD98059 or the vehicle controls (DMSO or methanol, used at the highest concentration present in any PD98059 treatment). In cells pretreated with PD98059, the virus was added directly to the culture medium (0.5% lamb serum in EMEM containing PD98059). In all experiments, once added, the DMSO, methanol, U0126, or PD98059 was kept in the medium throughout the course of the experiment.

RT assays. The assay for RT activity, used to measure virus production in culture supernatants, has been described previously (13).

Immunoprecipitations and Western blot analyses. Cells were washed once with ice-cold phosphate-buffered saline (PBS) containing 0.2 mM sodium orthovanadate and lysed at the indicated times in ice-cold lysis buffer (50 mM Tris

HCl [pH 7.7], 150 mM NaCl, 0.1% sodium dodecyl sulfate [SDS], 1% sodium deoxycholate, 0.5 mM NaF, 1% NP-40) supplemented with aprotinin (2 μ g/ml), 100 μ M leupeptin, and 1 mM sodium orthovanadate. Insoluble material was removed by centrifugation in an Eppendorf model 5415C centrifuge (14,000 rpm for 5 min), and protein concentrations of the resultant lysates were determined using the protein assay reagent from Bio-Rad (Hercules, Calif.). Equal amounts of protein were subjected to immunoprecipitation or Western blot analysis. For immunoprecipitations, typically 0.5 to 1 mg of whole-cell lysate was incubated overnight at 4°C with 2 to 4 μ g of the appropriate antiserum, after which protein A or G Sepharose (Amersham, Arlington Heights, Ill.) was added (1 h, 4°C) to capture the immune complexes. For Western blot analysis, typically 30 μ g of protein was separated by SDS-polyacrylamide gel electrophoresis (PAGE) and subjected to standard Western blotting protocols. Antiserum specific for MEK-1 was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, Calif.). Antiserum specific for ERK-1/2 was obtained from Upstate Biotechnology Inc. (UBI) (Lake Placid, N.Y.). Antiserum specific for activated MAPK (pMAPK) was obtained from Promega. Goat polyclonal antiserum raised against visna virus Gag was prepared in our laboratory previously.

***In vitro* kinase assays.** For MAPK kinase assays, immune complexes collected with protein A-Sepharose were washed three times in lysis buffer (defined above) and once in kinase buffer (50 mM Tris HCl [pH 7.7], 10 mM MgCl₂, 10 mM MnCl₂, 1 mM dithiothreitol), prior to incubation in an Eppendorf mixer for 15 min at room temperature in kinase buffer containing 10 μ g of myelin basic protein (MBP) (UBI) and 0.65 mCi of [γ -³²P]ATP (NEN Life Science Products Inc., Boston, Mass.) per ml. Samples were boiled in standard SDS-PAGE loading buffer, separated on a 12% gel, and analyzed by autoradiography. For assays of MEK-1 activity, MEK-1 immunoprecipitates were washed three times in lysis buffer (defined above), washed once in kinase buffer (defined above), and incubated at room temperature for 25 min in kinase buffer containing [γ -³²P]ATP (0.65 mCi/ml), 1.4 μ g of glutathione S-transferase (GST)-inactive ERK-2 (UBI), and 10 μ g of MBP. Active MEK-1 contained in the immunoprecipitates phosphorylates and activates GST-inactive ERK-2, which in turn phosphorylates MBP. Kinase products were separated by SDS-PAGE and analyzed by autoradiography.

Metabolic labeling of virions. Cells infected for 5 to 7 days with visna virus as described above were washed two times with Hanks' balanced salt solution (HBSS), starved in EMEM without Met for 1 h at 37°C, and labeled overnight at 37°C in EMEM without Met supplemented with 1% FBS and 70 to 150 μ Ci of Tran³⁵S-label (referred to throughout as ³⁵S-labeled) (ICN Pharmaceuticals, Irvine, Calif.) per ml. Culture supernatants containing virus were clarified twice by centrifugation at $1,200 \times g$ for 10 min, filtered through a 0.45- μ m-pore-size membrane, and concentrated by centrifugation through a 20% (wt/vol) sucrose cushion in TNE (25 mM Tris-HCl [pH 8.0], 150 mM NaCl, 2 mM EDTA) at $125,000 \times g$ for 1 h at 4°C. The virion pellets were lysed in 300 μ l of lysis buffer (defined above).

Analysis of viral entry. GSM and SCP cells plated and grown to approximately 90% confluence in 12-well culture plates were washed with EMEM–0.5% FBS, and medium was replaced with EMEM–0.5% FBS supplemented either with PD98059, with DMSO at concentrations present in drug-treated wells, or with EMEM–0.5% FBS for 1 h at 37°C. Cells were then infected for 3 h with visna virus (10^6 TCID₅₀ in EMEM–0.5% FBS supplemented with PD98059 or DMSO) that had been treated with RQ1 DNase (20 μ g/ml at 37°C for 30 min in the presence of 10 mM MgCl₂; Promega), and filtered (pore size, 0.45 μ m). Uninfected cells were treated exactly as infected cells except that EMEM–0.5% FBS alone was DNase digested, filtered, and put on wells with appropriate treatments. After 3 h, all wells were washed three times with EMEM–0.5% FBS (to remove virus) and medium (containing appropriate treatments) was replaced. The zero time point was defined as immediately after infection. At each time point (0 h and 7 h postinfection [p.i.]), cells were washed two times with PBS, scraped in 1 ml of PBS, and pelleted in a tabletop microcentrifuge. Cell pellets were lysed in 150 μ l of PCR lysis buffer (50 mM KCl, 10 mM Tris-HCl [pH 8.3], 1.5 mM MgCl₂, 0.001% gelatin, 0.45% NP-40, 0.45% Tween 20, and proteinase K [20 μ g/ml] [Boehringer Mannheim, Indianapolis, Ind.]) and incubated at 55°C for 90 min, followed by a 10-min incubation at 99°C to inactivate the proteinase K. Viral DNA was detected by PCR (5 min at 99°C, 45 s at 60°C, and 2 min at 72°C followed by 35 cycles of 1 min of denaturation at 94°C, 45 s of annealing at 60°C, and 45 s of extension at 72°C) on 25 μ l of lysate in a 50- μ l reaction mix with primers to the 5' end of the visna *gag* gene (5'-CTAGCTAGAGACATGGCG AAGC-3' and 5'-TAATGCCCATAGACAATTCCTT-3') or with primers to sheep glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (5'-TGCTGATG CCCTCATGTTTGTGATG-3' and 5'-GACATGGAAAGATATGCTCATGA GCT-3'). PCR products were separated on a 1.5% agarose gel.

RT-PCR. SCP cells plated and grown to approximately 90% confluence were washed with EMEM-0.5% FBS, and medium was replaced with EMEM-0.5% FBS supplemented either with PD98059, with methanol at concentrations present in drug-treated wells, or with EMEM-0.5% FBS for 1 h at 37°C. Cells were then infected for 6 h with visna virus (5×10^5 TCID₅₀ in EMEM-0.5% FBS supplemented with PD98059 or methanol). After 6 h, all wells were washed three times with HBSS to remove virus, and medium containing appropriate treatments was replaced. At the appropriate time points, all cells were washed vigorously in HBSS and RNA was extracted using RNA-STAT 60 (Tel-Test, Friendswood, Tex.). The procedure used for nuclear and cytoplasmic fractionation has been described previously (71). For cDNA synthesis, 5 µg of total cellular RNA or 1 µg of RNA from cell fractions was processed according to the manufacturer's instructions for Superscript II RT (Life Technologies). One tenth of the reaction was used as a template for PCR. The primer sequences and PCR conditions for *gag* and *GAPDH* are described above. The sequences of the *env* primers are 5'-GTCTCGGTGTCGAAACG-3' and 5'-GCCACGAGAACCAAGAGG-3'. PCR conditions for *env* were 35 cycles of 1 min of denaturation 94°C, 30 s of annealing at 51°C, and 45 s of extension at 72°C. The sequences of the *rev* primers are 5'-GCTAGATCTCCACCATGGCCAGCAAGAAAGTAAGCCAAGC-3' and 5'-TCGACGCGTTCACTATTAGTGCTTAAGCTTCGCAGCC-3'. PCR conditions for *rev* were 35 cycles of 1 min of denaturation 94°C, 45 s of annealing at 59°C, and 45 s of extension at 72°C. PCRs were separated on 1 to 1.5% agarose gels and analyzed using the Kodak Digital Science Image Station 440cf (Perkin-Elmer Life Sciences, Boston, Mass.). Band intensity was quantitated using Kodak 1D Image Analysis Software (version 3.5.3).

Immunohistochemical staining. To detect cells expressing pMAPK and MAPK, antibodies that detect pMAPK (Santa Cruz Biotechnology, Inc.) and MAPK (UBI) were used to immunohistochemically stain brain sections from visna virus-infected sheep with (S155) and without (S96) encephalitis. To determine whether pMAPK was expressed in astrocytes, tissues were immunohistochemically double labeled for pMAPK (above) and GFAP (polyclonal antiserum to bovine GFAP that cross-reacts with sheep GFAP; DAKO Corporation, Carpinteria, Calif.). To determine whether pMAPK was expressed in cells of macrophage lineage in the brain, pMAPK-stained tissues were histochemically stained with the lectin *Ricinus communis* agglutinin-1, which binds to macrophages, microglia, and endothelial cells in the brains of sheep. To ensure consistency and uniformity of staining, all samples were stained using an Optimax Plus automated cell stainer (BioGenex, San Ramon, Calif.). Briefly, Streck-fixed, paraffin-embedded tissue sections were deparaffinized and rehydrated and then postfixed in Streck tissue fixative for 20 min. For antigen retrieval, tissues were rinsed in water and heated in a microwave in sodium citrate (0.01 M, pH 6.0) for 8 min. Endogenous peroxidase was quenched with 3% H₂O₂ in water for 10 min, and then sections were blocked with buffered casein for 5 min. Primary antiserum was applied to the tissues for 60 min at room temperature, after which the tissues were washed in wash buffer (BioGenex), and secondary biotinylated multilink antibody (BioGenex) was applied for 20 min. The tissues were washed again, and streptavidin-horseradish peroxidase was added for another 20 min. The sections were washed again, and diaminobenzidine tetrahydrochloride in buffer containing H₂O₂ was applied to the sections for 10 min. The sections were washed, hematoxylin counterstained, dehydrated, and mounted. Double-labeled sections were not counterstained.

RESULTS

Visna virus infection activates MAPK in SCP and GSM cells. To examine the potential contribution of MAPK to visna virus replication, we first analyzed MAPK kinase activity in SCP or GSM cells infected or not with visna virus for 5 to 7 days. ERK-1/2 immunoprecipitates were assayed for kinase activity toward a classical MAPK substrate, MBP. The results demonstrate substantially augmented MAPK activity in virus-infected cells relative to the uninfected controls (Fig. 1). Importantly, we confirmed by Western blot analysis the ability of the antiserum raised against human ERK-1/2 to cross-react with sheep and goat ERK-1/2. These results confirmed that the increase in kinase activity did not result from increased MAPK expression (i.e., increased basal level MAPK activity [see below]).

FIG. 1. MAPK activity in SCP and GSM cells infected or not with visna virus for 5 to 7 days. ERK-1/2 immunoprecipitates were assayed for kinase activity toward MBP, separated by SDS-PAGE, and analyzed by autoradiography as described in Materials and Methods. The position of the MBP substrate is indicated on the right.

PD98059 inhibits visna virus replication. To evaluate the significance of MAPK activation to viral replication, we next examined the affect of PD98059 (a specific inhibitor of MEK-1/2, the direct upstream activators of MAPK) on virus-infected cultures. Virus production was assessed by measuring RT activity in supernatants from control or infected SCP or GSM cells in the presence or absence of PD98059 (effective doses determined empirically) or the appropriately diluted vehicle control (DMSO). The results indicate that supernatants from virus-infected cells treated with PD98059 contained markedly lower RT activity than supernatants from virus-infected untreated cells (Table 1). Of note, in some experiments moderate inhibition of virus replication occurred in the presence of DMSO alone; however, this effect was transient and never reached the inhibition levels achieved by treatment with PD98059. Nonetheless, to further evaluate the inhibitory effect of PD98059 on virus replication, the drug was solubilized in methanol and the experiments were repeated (Table 2). Supernatants from virus-infected cultures treated with PD98059 again contained substantially reduced RT activity compared to supernatants from infected cultures that were either untreated or treated with methanol. In addition, to exclude the possibility that the inhibitory effect was a nonspecific side effect of PD98059, we repeated the experiments using a different MEK-1/2-specific inhibitor, U0126 (Table 2). Treatment with U0126 also resulted in marked reduction of RT activity, thus confirming the ability of two chemically distinct MEK-1/2 inhibitors to suppress visna virus replication.

TABLE 1. Inhibition of visna virus replication by PD98059^a

Treatment	% Virus replication	
	SCP	GSM
None	100	100
DMSO	56.4 ± 3.5	83.0 ± 30.0
PD98059 ^b	18.8 ± 7.1	4.7 ± 0.9

^a Virus replication was assessed by measuring RT activity (in counts per minute per milliliter) in supernatants of infected SCP or GSM cells treated or not with DMSO or PD98059. Treatments were added 1 h prior to infection and maintained in the media throughout the experiment. Supernatants were collected after 5 to 7 days of infection. Data are expressed as a percentage of the infected, untreated samples. Data for treated samples are means ± standard deviations.

^b SCP cells were treated with 50 µM PD98059 and GSM cells were treated with 25 µM PD98059.

TABLE 2. Inhibition of visna virus replication by PD98059 and U0126^a

Treatment	% Virus replication
None	100
Methanol	83.9 ± 15.5
PD98059 ^b	2.7 ± 0.8
DMSO	92.5 ± 21.2
U0126 ^c	0.4 ± 0.6

^a Virus replication was assessed by measuring RT activity (in counts per minute per milliliter) in supernatants of infected SCP cells treated or not with DMSO, methanol, PD98059, or U0126. Treatments were added 1 h prior to infection and maintained in the media throughout the experiment. Supernatants were collected after 5 to 7 days of infection. Data are expressed as a percentage of the infected, untreated samples. Data for treated samples are means ± standard deviations.

^b SCP cells were treated with 50 μM PD98059 solubilized in methanol.

^c SCP cells were treated with 50 μM U0126 solubilized in DMSO.

We next considered the possibility that PD98059 inhibits the enzymatic activity of RT directly or virion incorporation of RT (but not the actual production of virus particles), thereby facilitating misinterpretation of the data obtained solely from our RT-based assessment of viral replication. To address this potential scenario, we took two independent approaches. In the first approach, relevant doses of PD98059 were added directly to the RT enzymatic reaction of a known positive control sample. The results indicated that 25, 50, and 100 μM PD98059-treated samples yielded RT activity (111,452, 173,636, and 151,899 cpm/ml, respectively) closely resembling that of the untreated positive control (139,250 cpm/ml), demonstrating that PD98059 does not directly inhibit RT activity. Similar results were obtained with U0126 (data not shown). In the second approach, we analyzed Gag protein levels in supernatants derived from ³⁵S-labeled control or infected cells treated or not with PD98059. Gag immunoprecipitates were prepared from virus isolated from the supernatants by ultracentrifugation and analyzed by SDS-PAGE (Fig. 2). Supernatants derived from untreated or methanol-treated infected SCP cells contained similar and abundant Gag levels, whereas supernatants derived from PD98059-treated infected cells con-

FIG. 2. Analysis of Gag protein levels in culture supernatants. Virus pelleted from supernatants of ³⁵S-labeled uninfected SCP cells (Mock), infected untreated SCP cells (Visna), or infected SCP cells treated with methanol (Visna/MeOH) or 25 μM PD98059 (Visna/PD98059) was lysed and used to prepare immunoprecipitates of viral Gag which were separated by SDS-PAGE and analyzed by autoradiography as described in Materials and Methods. Treatments were added 1 h prior to infection and maintained in the media throughout the experiment. Results are representative of three independent experiments.

FIG. 3. (A) MEK-1 kinase assay. In vitro kinase assays were performed on MEK-1 immunoprecipitates prepared from control cells (Mock) or visna virus-infected SCP cells (Visna) treated or not with 50 μM PD98059 (Visna-PD). In the assay, active forms of MEK-1 contained in immunoprecipitates phosphorylate and thereby activate GST-inactive ERK-2, which in turn phosphorylates the substrate, MBP. (B) Western blot analysis of MAPK. Whole-cell lysates prepared from visna virus-infected GSM cells treated or not with DMSO or the indicated doses of PD98059 were separated by SDS-PAGE and processed for Western blot analysis using antiserum specific for ERK-1/2. Treatments were added 1 h prior to infection and maintained in the media throughout the experiment. Cell lysates were processed after 5 to 7 days of infection as described in Materials and Methods.

tained barely detectable levels of Gag. Collectively, these data suggest that the inhibitory effect of PD98059 on virus replication is manifested at a cellular level, which ultimately results in profound reduction of progeny virions released into the culture supernatant.

PD98059 inhibits MEK-1 and MAPK activity in ovine and caprine cells. The results presented above provide strong evidence to support the hypothesis that visna virus-induced activation of MAPK is required for viral replication. However, because these data were derived from experiments performed in ovine and caprine cells, as opposed to the human or murine cells that are routinely used as model eukaryotic cells, we wanted to confirm the ability of PD98059 to inhibit virus-induced activation of the MEK-1/MAPK pathway in SCP and GSM cells. To this end, MEK-1 immunoprecipitates prepared from control or visna virus-infected SCP cells treated or not with PD98059 were examined in in vitro kinase assays. The basis of the assay is as follows: activated MEK-1 (contained in the immunoprecipitates) phosphorylates and thereby activates GST-inactive ERK-2, which in turn phosphorylates the substrate MBP. The results (Fig. 3A) demonstrate substantially increased MEK-1 activity in visna virus-infected SCP cells, which is essentially abolished by treatment with PD98059, confirming the specificity of PD98059 for sheep-derived MEK-1. Unfortunately, the MEK-1 antiserum did not cross-react with GSM cell lysates either in kinase assays or Western blots. Therefore, we assessed the effectiveness of PD98059 in GSM cells by analyzing MAPK activation in virus-infected GSM cells treated or not with PD98059. The results (Fig. 3B) demon-

FIG. 4. Western blot analysis of phosphotyrosine and ERK-1/2. Whole-cell lysates were prepared from mock-infected SCPs (MOCK) or SCPs infected for the indicated times with visna virus (V) isolated from cell culture supernatants by ultracentrifugation through a sucrose cushion as described in Materials and Methods. Equal amounts of protein were separated by SDS-PAGE and processed for Western blot analysis using antiserum specific for phosphotyrosine (PT) (top panel) or ERK-1/2 (bottom panel).

strate a dose-dependent inhibition of MAPK activity by PD98059, as faster-migrating MAPK (dephosphorylated or inactive) appears optimally in cells treated with 25 μ M PD98059. Note that the amount of MAPK present in each lane is not different; only the activation state is affected by PD98059, demonstrating that PD98059 acts in caprine-derived cells analogously as described previously in human and murine cells. Accordingly, we conclude from the cumulative data that activated MAPK plays an essential role in the replication of visna virus.

Visna virus rapidly activates MAPK. Given that activated MAPK is required for viral replication, it follows that virus infection must induce timely activation of the MAPK pathway in order to complete the replication cycle. Results from two previous reports support the ability of visna virus to activate cellular signaling pathways. In the first report, U937 cells transiently transfected with an expression vector encoding visna virus Tat resulted in enhanced expression of *c-jun* mRNA (59). Because visna virus Tat cannot bind DNA, the authors concluded that the increase in *c-jun* mRNA resulted from activation of a cellular signaling pathway. In the second report, antiserum raised against a cellular membrane protein bound by visna virus, also able to inhibit virus binding to membranes and virus infection (10, 20), was found to immunoprecipitate a serine/threonine kinase complex (4). Based on the latter report, we considered the possibility that the virus attachment step might trigger initial MAPK activation. Using MAPK activation induced by traditional membrane receptor signaling as a temporal guide, whole-cell lysates were prepared 15, 30, and 45 min postinoculation with visna virus. Of note, the virus used in these experiments had been isolated from culture supernatants by ultracentrifugation through a sucrose cushion since supernatants of visna virus-infected cells likely contain cytokines and chemokines that may activate MAPK. Western blots were prepared from products of SDS-PAGE separation of equal amounts of protein and probed with either antiphosphotyrosine antibodies or anti-ERK-1/2 antibodies. The results of antiphosphotyrosine blots (Fig. 4, top panel) demonstrate a rapid increase in phosphotyrosine content of 44- and 42-kDa

FIG. 5. PCR to measure newly synthesized viral *gag* DNA in PD98059-treated virus-infected cells. SCP or GSM cells were incubated for 1 h with EMEM-0.5% FBS supplemented with PD98059 (50 or 25 μ M, respectively) (D), DMSO (V), or no treatment (M) and then infected (+) or not (-) with visna virus for 3 h as described in Materials and Methods. Cell lysates prepared at the time of infection ($t = 0$) or 7 h p.i. were used in PCRs with primers specific for the 5' portion of the visna *gag* gene or with primers for sheep GAPDH. A no-template PCR was done for each set of primers (neg. control). Products were separated on a 1.5% agarose gel.

proteins, which exhibit the subtle retarded migration characteristic of activated ERK-1/2 (44 and 42 kDa, respectively). A parallel blot probed with anti-ERK-1/2 antibodies (Fig. 4, bottom panel) confirmed the rapid activation of MAPK again evidenced by the characteristic upward electrophoretic shift. Lines depicting the lower limits of ERK-1/2 bands have been added to facilitate examination of the shifted bands (Fig. 4, bottom panel). These results indicate that virus-induced activation of the MAPK pathway occurs very early in the infection process.

PD98059 does not inhibit viral entry. In order to determine the stage of virus replication inhibited by PD98059, we first examined the effect of PD98059 on viral entry. To measure early events of virus entry, we used PCR to detect newly synthesized viral DNA. SCP and GSM cells were treated with PD98059 or appropriate controls for 1 h and infected with visna virus in the same treatments for 3 h, and time points were taken at the time of visna virus addition to the cells ($t = 0$) and at 7 h p.i. Primers specific for the 5' end of the visna *gag* gene were used in PCRs on DNA isolated from the infected and uninfected drug-treated and control cells. To control for relative amounts of DNA at each time point, sheep GAPDH primers were used to ensure that the differences in *gag* PCR products were not due to differences in total DNA concentrations. There was no discernible effect of PD98059 on the production of newly synthesized viral *gag* DNA at 7 h p.i. (Fig. 5), suggesting that PD98059 is not inhibiting entry of visna virus into cells or the early steps of reverse transcription.

PD98059 inhibits the expression of *gag* mRNA and protein. Based on the results shown in Fig. 2, illustrating extremely low Gag protein levels in supernatants from PD98059-treated virus-infected cells, we next examined Gag protein levels present in cells treated analogously, to provide some indication of their ability to produce viral proteins. Gag protein expression in virus-infected SCP cells treated or not with PD98059 was analyzed by Western blot analysis (Fig. 6A). The results demonstrate a marked decrease in Gag protein expression that parallels a dose-dependent increase in PD98059 concentration.

Because low Gag protein expression may simply reflect the inability of virus to spread throughout the culture, we examined *gag* mRNA levels in SCP cells early after infection with a 10-fold-higher TCID₅₀ (5×10^5 TCID₅₀) to maximize infection (Fig. 6B). The results of RT-PCR analysis indicate that cells treated with PD98059 contain lower levels of *gag* mRNA than untreated or DMSO-treated cells, as early as 1 day p.i. Quantitation of band intensity revealed that *gag* mRNA levels in PD98059-treated cells are three- to fivefold lower than those in control cells by 2 days p.i. (Fig. 6C). There was no effect of PD98059 on cellular GAPDH mRNA expression, which is not surprising given that MAPK is not activated in these cells prior to infection and hence is not a requirement for GAPDH expression, growth, or viability of SCP cells in culture.

To evaluate if the decrease in *gag* mRNA was *gag*-specific or if the expression of other viral genes may also be decreased, we examined levels of singly spliced *env* and multiply spliced *rev* mRNA in infected SCP cells treated with PD98059 (Fig. 6D and E). The results indicate that *env* mRNA is decreased to levels similar to those observed for *gag*, while *rev* mRNA levels appear comparatively unaffected by treatment with PD98059. Collectively, these data enable us to make the following conclusions. (i) Because viral transcription is dependent on integration (45) and because *rev* mRNA levels are not substantially altered by treatment with PD98059, MAPK activation is not required for virus integration or transcription. (ii) Because PD98059 markedly affects only the expression of unspliced (*gag*) and singly spliced (*env*) mRNA, these data bear a striking resemblance to viral mRNA expression in the presence of a dysfunctional Rev protein (35, 47), suggesting a mechanism wherein lack of activated MAPK precludes visna virus Rev function.

PD98059 inhibits the cytosolic expression of *gag* and *env* but not *rev* mRNA. The classic phenotype associated with Δ rev and various mutant *rev* lentiviruses is decreased cytosolic expression of unspliced (*gag*) or singly spliced (*env*) mRNA (40, 62). To determine if PD98059 inhibits the nucleocytoplasmic transport of *gag* and *env* mRNA, RT-PCR was performed on RNA isolated from nuclear and cytosolic fractions of SCP cells infected with visna virus (5×10^5 TCID₅₀) for 2 days. The results (Table 3) indicate that cytosolic expression of *gag* and *env* mRNA was markedly reduced in the presence of PD98059, while nuclear expression of these transcripts was either unal-

FIG. 6. (A) Western blot analysis of Gag expression in control or virus-infected (between 1×10^4 TCID₅₀ and 5×10^4 TCID₅₀) SCP cells that were untreated or treated with DMSO or the indicated doses of PD98059. Cell lysates were processed after 5 to 7 days of infection as described in Materials and Methods. (B) RT-PCR of *gag* and GAPDH mRNA in PD98059-treated virus-infected (5×10^5 TCID₅₀) SCP cells. (C) Graphical representation of band intensities depicted above in panel B. Graphed values represent *gag* band intensities that have been normalized to GAPDH band intensities. (D) RT-PCR of *env* and *rev* mRNA in PD98059-treated virus-infected (5×10^5 TCID₅₀) SCP cells. (E) Graphical representation of band intensities depicted above in panel D. Graphed values represent *env* and *rev* band intensities that have been normalized to GAPDH band intensities. Treatments were added 1 h prior to infection and maintained in the media throughout the experiment. RNA was processed after 1, 2, and 3 days of infection as described in Materials and Methods. Results are representative of several independent experiments.

TABLE 3. PD98059 suppresses cytoplasmic expression of *gag* and *env* mRNA^a

Sample	% Band intensity (mean $\pm$ SD)	
	MeOH	PD98059
<i>gag</i>		
Nuclear	82.9 $\pm$ 8.3	92.9 $\pm$ 22.46
Cytoplasmic	86.0 $\pm$ 2.78	50.8 $\pm$ 6.8
<i>env</i>		
Nuclear	119.1 $\pm$ 27.9	114.0 $\pm$ 18.6
Cytoplasmic	89.5 $\pm$ 13.0	53.4 $\pm$ 6.6
<i>rev</i>		
Nuclear	61.7 $\pm$ 11.8	70.4 $\pm$ 6.6
Cytoplasmic	111.5 $\pm$ 0.7	128.0 $\pm$ 14.2

^a Relative band intensities of RT-PCR of *gag*, *env*, and *rev* mRNA isolated from nuclear or cytoplasmic fractions of SCP cells infected with visna virus (5×10^5 TCID₅₀). Infected cells were treated or not with methanol (MeOH) or 50 μ M PD98059. Data are expressed as a percentage of the infected, untreated samples in each fraction (untreated samples thus had an intensity of 100%). Treatments were added 1 h prior to infection and maintained in the media throughout the experiment.

tered (*gag*) or increased (*env*) compared to samples treated with the vehicle control. These results are consistent with previous observations of unspliced and singly spliced viral mRNA levels in the absence of Rev function (26, 47).

In contrast, the cytoplasmic expression of *rev* mRNA was not reduced but rather increased in the presence of PD98059, again consistent with previous observations regarding the expression of fully spliced mRNAs in the absence of Rev function (26, 47). The nuclear expression of *rev* mRNA fluctuated between 25 and 35% decreased expression, which may reflect to some extent the unique competition of *rev* primers for the abundant unspliced, singly spliced, and multiply spliced (*rev*) mRNAs in the nucleus but is nonetheless in keeping with the Rev independence of *rev* mRNA expression (40). In any event, that cytosolic expression of unspliced and singly spliced, but not multiply spliced, viral mRNA is substantially decreased in the presence of PD98059 confirms our earlier suspicions that optimal Rev function is dependent on activated ERK-1/2.

PD98059 inhibits visna virus replication in primary sheep macrophages. Although SCP and GSM cells are the classical cells used to study visna virus replication in vitro, macrophages are the predominant target cells for visna virus infection in vivo (32, 33, 58, 77, 78, 91). As the first step toward evaluating the potential relevance of our in vitro findings (above), we examined the effect of various doses of PD98059 on visna virus replication in primary sheep macrophages. Supernatants derived from virus-infected sheep macrophages treated with 0, 12.5, 25, 50, and 100 μ M PD98059 contained 6,673, 2,155, 1,585, 842, and 934 cpm of RT activity per ml, respectively. These results demonstrate a dose-dependent inhibition of virus production, similar to the primary cell lines described above, and prompted further investigation regarding virus-induced activation of MAPK in vivo.

MAPK is activated in CNS macrophages and astrocytes. CNS disease is one of the classical pathologies associated with lentivirus infection (91). As such, many studies are in progress with the goal of defining the molecular determinants of lentivirus neuropathogenesis. We have focused primarily on simian

immunodeficiency virus and visna virus, and using in vivo passage, we have developed neurovirulent strains of both lentiviruses that reproducibly cause CNS disease (19, 75). The pathological changes of visna virus encephalitis are characterized by intense periventricular and perivascular infiltrates of macrophages and lymphocytes and multifocal demyelination, accompanied by substantial astrocytic hyperplasia with marked upregulation of GFAP expression (89). Interestingly, a recent study suggested that ERK/MAPK is chronically active in human reactive astrocytes and proposed the hypothesis that activation of the ERK/MAPK pathway is an obligatory step for the triggering and/or persistence of reactive astrogliosis (49). In light of this report and the results of our in vitro experiments demonstrating the importance of activated MAPK to visna virus replication, we next examined activation of MAPK in brains of visna virus-infected sheep.

To determine whether MAPK activation is characteristic of visna virus-induced encephalitis, we stained the brains of infected sheep with (S155) and without (S96) encephalitis immunohistochemically with antibodies to MAPK and pMAPK (specific only for activated MAPK). The results indicated a marked upregulation of expression of pMAPK in the brains of sheep with encephalitis (Fig. 7b); specifically, pMAPK was expressed in cells in the perivascular cuffs and in periventricular white matter, areas where there are abundant infiltrating macrophages and activated astrocytes. In contrast, only rare cells expressing pMAPK were identified in the quiescent brains of sheep without encephalitis (Fig. 7a). Of note, there was no visible difference in overall MAPK expression in the brains of animals with or without encephalitis (Fig. 7c and d). These results were also confirmed by Western blot analysis of tissue homogenates prepared from these animals and two additional sheep, one with encephalitis and one without (not shown).

To identify the cells expressing pMAPK in the brains of sheep with encephalitis, we performed double-labeling immunohistochemistry experiments using markers of macrophages and astrocytes in combination with antiserum specific for pMAPK. There was clear colocalization of pMAPK with RCA-1, a marker of macrophages and brain microglia, indicating upregulation of pMAPK in cells of macrophage lineage in the brain (Fig. 7e). pMAPK expression also colocalized with expression of GFAP, a marker of astrocytes, indicating upregulation of pMAPK in astrocytes (Fig. 7f). These results suggest that activation of MAPK correlates with visna virus-induced neuropathology and that, in addition to microglia, activated MAPK is present in astrocytes, cells not considered to be major targets of visna virus replication in the CNS.

DISCUSSION

The infidelity of lentivirus replication facilitates acquisition of the molecular resistance required to survive selective pressures imposed by current antiviral agents. Moreover, as shown for HIV, latent reservoirs can harbor drug-resistant virus that confounds treatment management (66). Hence, the efficacy of long-term antiviral therapy warrants continued development of alternative or complementary therapeutic strategies. One approach involves the identification of essential virus-cell interactions that provide direct and novel therapeutic targets. In addition, because some pathological changes associated with

lentivirus infection share common features with other diseases, such as HIV-associated dementia and Alzheimer's disease (7, 80), delineation of cellular mechanisms forecasting or strongly associated with pathological onset of disease may expose tissue-specific processes responsive to therapeutic intervention (49, 72, 90).

Visna virus, a lentivirus originally discovered in the 1950s, has long been a cause of debilitating disease in sheep, resulting in significant economic losses in livestock industries (60). The CNS disease associated with visna virus infection is characterized by intense periventricular and perivascular infiltrates of macrophages and lymphocytes, multifocal demyelination, and reactive astrogliosis (91). The studies presented in this report provide evidence that visna virus induces and sustains activation of ERK-1/2, which is, in turn, required for productive virus replication. Moreover, the presence of activated ERK-1/2 in brain cells, identified both as classical targets for visna virus replication *in vivo* (macrophages/microglia) as well as cells not known to be susceptible (astrocytes), correlates strongly with virus-induced encephalitis. In this regard, visna virus-induced encephalitis resembles the chronic activation of ERK-1/2 observed in humans exhibiting reactive astrogliosis in response to infarct, mechanical trauma, chronic epilepsy, and progressive multifocal leukoencephalopathy (49). In a broad context, these similarities may reflect a common pathological mechanism and support the hypothesis suggested by Mandell and VandenBerg that activation of the ERK/MAPK pathway is an obligatory step for the triggering and/or persistence of reactive astrogliosis (49). In a more focused context, the requirement of activated ERK-1/2 for visna virus replication is significant with regard to our current understanding of cellular proteins and pathways required for lentivirus replication and associated neurodegenerative pathology.

To evaluate the importance of ERK/MAPK activation to visna virus replication, we used PD98059 as a potent and specific inhibitor of the MEK/ERK pathway. The first detectable effect of PD98059 on virus replication manifested in decreased cellular expression of *gag* and *env* mRNA, but not *rev* mRNA. Because there was essentially very little change in total cellular *rev* mRNA, these results ruled out profound defects in viral integration and transcription (45). Further examination demonstrated that decreased cellular expression of *gag* and *env* mRNA reflected decreased cytoplasmic rather than nuclear expression of these viral mRNAs, highly suggestive of a MAPK-dependent defect in Rev function. Rev is an essential

lentivirus protein with a highly conserved function that shuttles between the nucleus and cytoplasm and is responsible for the nucleocytoplasmic transport of all unspliced and singly spliced viral mRNAs (40, 62). In the absence of Rev function, viral mRNAs containing introns are either rapidly degraded or spliced to completion, resulting in a viral mRNA expression pattern exactly as described above for PD98059-treated cells infected with visna virus; namely, decreased cytosolic expression of unspliced (*gag*) and singly spliced (*env*) mRNA while nuclear expression appears largely unaffected (26, 47). Also consistent with our results using PD98059 (Fig. 6; Table 3), others have found that the expression of singly spliced viral mRNA may be slightly less dependent on Rev than the expression of unspliced viral mRNA (47, 69). In addition, our results that cytosolic expression of *rev* mRNA is largely unaffected or even increased in the presence of PD98059 is consistent with observations of multiply spliced mRNA expression in the absence of Rev function (25, 26, 35, 47, 48), further strengthening the hypothesis that PD98059 affects Rev function.

Three primary functions of the lentivirus Rev proteins (outlined below) have been identified and mapped to specific amino acid domains in Rev (reviewed in references 40 and 62). The first functional domain (typically arginine rich) is responsible for nuclear localization as well as binding to the Rev responsive element (RRE) present in unspliced and singly spliced viral mRNAs. Closely associated with this domain, but distinct, are amino acids required for multimerization, another essential feature of Rev function. Finally, there is an activation domain (typically leucine rich) that is responsible for nuclear export of Rev-associated mRNAs. Mutations in this domain have a dominant negative phenotype. Additional studies are required to evaluate the effect of PD98059 on each of the classical functions of Rev, as well as other suggested functions such as mRNA stabilization (26, 47) and enhanced polysome loading (3, 21). However, if PD98059 affects only polysome loading, we would have anticipated unaltered cytosolic levels of *gag* and/or *env* mRNA rather than the decreased levels we consistently observed.

That inhibition of ERK-1/2 simulates a Δ Rev phenotype makes it tempting to speculate on the involvement of Rev phosphorylation in Rev function. Like other lentivirus Rev proteins (37, 71), visna virus Rev (VV-Rev) is a phosphoprotein, although perhaps to a lesser extent (70). Unlike HIV type 1 (HIV-1) Rev, the most highly studied lentivirus Rev protein, analysis of the VV-Rev amino acid sequence reveals no con-

FIG. 7. Immunohistochemical analysis of white matter from the brains of visna virus-infected sheep with (S155) or without (S96) encephalitis. Compare panels a and b and panels c and d. (a) White matter from the brain of sheep S96 stained immunohistochemically for pMAPK (activated MAPK). Expression of pMAPK is very low, with only rare positive cells within the brain parenchyma (arrow). (Magnification, $\times 95$; hematoxylin counterstain.) (b) White matter from the brain of sheep S155 stained immunohistochemically for pMAPK. Numerous cells are expressing pMAPK, including perivascular mononuclear inflammatory cells (arrow) and scattered cells throughout the parenchyma. (Magnification, $\times 95$; hematoxylin counterstain.) (c) White matter from the brain of sheep S96 stained immunohistochemically for MAPK. Numerous cells throughout the parenchyma are expressing MAPK. (Magnification, $\times 95$; hematoxylin counterstain.) (d) White matter from the brain of sheep S155 stained immunohistochemically for MAPK. Numerous cells throughout the parenchyma are expressing MAPK. (Magnification, $\times 95$; hematoxylin counterstain.) (e) White matter from the brain of sheep S155 double labeled by immunohistochemistry with antiserum against pMAPK (blue precipitate) and by lectin histochemistry with the peroxidase-conjugated lectin *R. communis* agglutinin-1, which detects brain microglia (brown precipitate). A double-labeled cell is shown (arrow), indicative of a microglial cell expressing pMAPK. (Magnification, $\times 380$; no counterstain.) (f) White matter from the brain of sheep S155 double labeled immunohistochemically for pMAPK (blue precipitate) and the astrocyte marker GFAP (brown precipitate). Several double-labeled cells are shown (arrows), indicating expression of pMAPK by astrocytes. (Magnification, $\times 190$; no counterstain.)

sensus ERK-1/2 phosphorylation sites; however, potential phosphorylation by ERK-1/2-activated kinases cannot be ruled out. Despite the many reports demonstrating that HIV-1 Rev can be phosphorylated by CKII and MAPK in vitro (50, 51, 88), only a single report implicating the relevance of phosphorylation to Rev function has emerged and suggests that HIV-1 Rev phosphorylation accelerates formation of an efficient RNA-binding conformation (28). No analogous study has been reported regarding VV-Rev, although it is clear that VV-Rev can rescue expression of HIV-1 structural proteins from an HIV-1 Δ rev proviral clone, if the HIV-1 RRE is replaced with the visna virus RRE (VV-RRE) (83). These results demonstrate that HIV-1 Rev and VV-Rev are functionally equivalent provided access of VV-Rev to VV-RRE, a finding consistent with other studies demonstrating the inability of HIV-1 Rev to bind VV-RRE and vice versa (84). Supporting the concept that these Rev proteins act through a similar mechanism, other studies have shown that chimeric proteins that express the binding domain of HIV-1 Rev fused to the activation domain of VV-Rev (or vice versa) are fully functional in the context of the sequence requirements (for RRE) dictated by each respective binding domain (85). Interestingly, however, in a report demonstrating that the activation domains of HIV-1 and VV-Rev proteins contain nuclear export signals (NES), Meyer et al. noted that twice as much of an HIV-1 NES competitor was required to inhibit the visna virus NES compared to the HIV-1 NES (52). The authors suggested that the VV-Rev activation domain may bind a common cofactor more efficiently than the HIV-1 Rev activation domain or that the VV-Rev activation domain may access alternative cofactors that can functionally replace those competed away by the HIV-1 Rev activation domain. Hence, the activation domains of VV-Rev and HIV-1 Rev may not function 100% identically. Accordingly, it is possible that an alternative cofactor required for optimal VV-Rev function is regulated by ERK-1/2 and in retrospect, it would have been interesting to know the activation states of ERK-1/2 during these earlier studies. Indeed, it is difficult to interpret our present results in the context of previously published observations (regarding the well-studied HIV-1 Rev protein and the interchangeability of HIV-1 and visna virus Rev proteins) without knowing the activation states of ERK-1/2 in each of the experimental systems. As such, and in light of our present findings, it seems appropriate to evaluate the interchangeability of VV-Rev and HIV-1 Rev function in the presence of PD98059.

Relevantly, the ERK/MAPK pathway activated and sustained by visna virus infection is only transiently (~5 min) activated by HIV-1 infection (via activation of CD4/CXCR4; [63]) and, thus, is not likely a requirement for HIV-1 Rev function. This transient ERK-1/2 activation appears to promote activation of NF- κ B and AP-1, thereby optimizing the kinetics of virus replication (8). Of interest, other studies have suggested that HIV-1 replication may in fact rely on activation of a distinct MAPK family member, p38, as sustained activation of p38 was observed in infected primary T lymphocytes (17). Moreover, inhibitors of p38 significantly reduced virus replication both in infected T lymphocytes and peripheral blood mononuclear cells (17, 74). Intriguingly, both ERK/MAPK and p38/MAPK have been shown to play active roles in posttranscriptional regulation. Specifically, ERK-1/2 has been

implicated in nucleocytoplasmic transport of mRNA (24), and p38 has been implicated in mRNA stabilization (68), both strikingly and unavoidably purported functions of Rev (26, 47). In addition, both MEK-1/2 (1) and p38 (34) have been shown to regulate nucleocytoplasmic transport of cellular proteins via pathways linked through sensitivity to leptomycin B to CRM1, a nuclear export receptor (27, 30, 82). Significantly, CRM1 is one of the essential cofactors of HIV-1 Rev-dependent function (87). Hence, the possibility exists that either or both MAPK pathways may contribute to Rev function.

Activation of MAPK by viruses is not unique to lentiviruses and extends to other viruses, including herpes simplex virus type 2 (79), hepatitis B viruses (5), hepatitis C virus (38), echovirus 1 (43), friend spleen focus-forming virus (55), borna disease virus (36), respiratory syncytial virus (53), human cytomegalovirus (67), simian virus 40 (81), coxsackievirus B3 (42), and adenovirus (9). Infection with influenza virus also activates and sustains activation of ERK-1/2 (61). Intriguingly, inhibition of influenza virus-induced activation of ERK-1/2 leads to inhibition of virus replication due to nuclear retention of viral ribonucleoprotein complexes and impaired function of the viral nuclear export protein NEP/NS2 (61). Hence, the concerted requirement of activated ERK-1/2 for nucleocytoplasmic transport of viral RNA, by two viruses (visna virus and influenza virus) with diverse replication strategies, may underscore a common mechanism of virus replication and reveal a vulnerable therapeutic target for viruses dependent on such an RNA transport vehicle. In this regard, recent studies are promising with regard to the in vivo tolerance of PD184352, an orally active MEK inhibitor (72) that may ultimately prove beneficial for the treatment of human and animal viral pathogens and/or accompanying neuropathologic sequelae.

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