Brief Definitive Report

Stable Expression of Transdominant Rev Protein in Human T Cells Inhibits Human Immunodeficiency Virus Replication

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Summary

The human immunodeficiency virus (HIV) Rev protein is essential for viral structural protein expression (Gag, Pol, and Env) and, hence, for viral replication. In transient transfection assays, mutant forms of Rev have been identified that inhibit wild-type Rev activity and therefore suppress viral replication. To determine whether such transdominant Rev proteins could provide long-term protection against HIV infection without affecting T cell function, T leukemia cell lines were stably transduced with a retroviral vector encoding a transdominant mutant of the Rev protein, M10. While all the M10-expressing cell lines remained infectable by HIV-1, these same cells failed to support a productive replication cycle when infected with a cloned isolate of HIV-1. In addition, two out of three M10-expressing CEM clones were also resistant to highly productive infection by a heterogeneous HIV-1 pool. Expression of M10 did not affect induction of HIV transcription mediated by the κB regulatory element or Tat. Importantly, constitutive expression of Rev M10 did not alter the secretion of interleukin 2 in response to mitogen stimulation of EL-4 and Jurkat cells. The inhibition of HIV infection in cells stably expressing a transdominant Rev protein, in the absence of any deleterious effect on T cell function, suggests that such a strategy could provide a therapeutic effect in the T lymphocytes of acquired immunodeficiency syndrome patients.

Replication of human retroviruses, unlike that of their avian and murine counterparts, is dependent upon virally encoded transactivator proteins. These proteins function by specifically modulating host cell processes and must, therefore, interact with host cell factors. One approach to the inhibition of retroviral infection is to express transdominant forms of viral transactivators that interfere with their function (1, 2). However, should inhibition be achieved by the interaction of the mutant protein with a factor essential for normal cell function, then such a strategy becomes contraindicated.

The HIV-1 Rev protein is encoded by a fully spliced viral mRNA synthesized early in virus infection (3). Rev activates the cytoplasmic expression of unspliced viral mRNAs (4–7), presumably interacts with host cell factors (8–11), and may be important for regulating virus latency (11, 12). Towards the COOH terminus of Rev, there is a well-conserved leucine-rich domain that is absolutely essential for function (13–16). Mutations in this region give rise to defective proteins that are transdominant inhibitors of Rev function in transient transfection assays (13–16). In vitro analyses have suggested that inhibition occurs by the formation of nonfunctional heteromeric Rev multimers unable to interact with the host cell factors required for the cytoplasmic expression of unspliced viral mRNAs (17). Together, these observations suggest that the expression of transdominant negative mutants of Rev may represent a potential strategy for anti-HIV therapy. In this report, the effects of stable expression of a Rev transdominant, M10, in leukemic T cell lines are examined.

Materials and Methods

Preparation of Retroviral Vectors and Cell Transduction. The PLJ plasmid (18) was digested with BamHI, filled in with Klenow, and digested with SalI. This fragment was ligated to a 592-bp insert derived from GST/M10 (17), prepared by digestion with NcoI, incubation with Klenow enzyme, followed by digestion with XhoI. The PLJ cRev M10 plasmid was introduced by calcium phosphate transfection into the ecotropic ψCRE virus-packaging cell line (19).

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Filtered ψCRE supernatant collected after 2 d was used to infect the amphotropic ψCRIP-packaging line, which was then selected in G418 (1.0 mg/ml). 24 clones were analyzed by titration for G418 resistance, and three producer clones were selected with titers of $\sim 10^5$/ml. Viral supernatants were incubated with CEM, Jurkat, and EL-4 cells (5×10^5/ml) for 4–8 h each day on four consecutive days, with cells maintained otherwise in RPMI 1640 with 10% FCS. Cells were selected in G418 (1.0 mg/ml) and subclones of G418-resistant CEM cells were isolated by limiting dilution. Subclones were tested for PLJ-cREV M10 provirus by Southern blot analysis by digesting high molecular weight DNA with XbaI and probing for a 4.49-kb fragment with the 592-bp cREV M10 insert. As a control, CEM cells were transduced with a β-galactosidase retroviral vector that confers G418 resistance, BAG, as previously described (20, 21).

Construction of pIIIB/CAT. The replication-competent, chloramphenicol acetyltransferase (CAT)-expressing proviral clone, pIIIB/CAT, is similar in design to vectors described previously by Terwilliger et al. (22). In pIIIB/CAT, the CAT gene has replaced the 5′ portion of the viral *nef* gene and is therefore, like Nef, expressed independent of Rev. To construct this vector, an NcoI site was introduced at the initiation codon of *nef* in the pIIIB proviral clone (23) by targeted mutagenesis (AGATGG→ACCATGG). A 681-bp NcoI-Asp718 CAT gene fragment was generated by digesting the products of a PCR that had been performed with primers designed to introduce these sites at the 5′ and 3′ ends of CAT. This fragment was inserted between the introduced NcoI site and the Asp718 site (HXB-3 isolate, sequence coordinates 8374 and 8593, respectively) 70 bp upstream of the 5′ boundary of the 3′ LTR U3 region. To partially compensate for the increased length of the virus genome, the sequences of the 3′ LTR U3 region between the EcoRV sites (sequence coordinates 8698 and 8998) were deleted. The net increase in genome length is therefore only 163 nucleotides.

Cell Culture and HIV Infection. CEM subclones were infected with 0.5 ml of filtered virus supernatant derived from highly productive infections of CEM cells with the pIIIB or pIIIB/CAT molecular clones (23), or H9 cells with the uncloned, heterogeneous HTLV-IIIB isolate (24). Cells were maintained at $0.5–2.5 \times 10^6$ cells/ml in RPMI 1640 containing 10% FCS. Virus replication was monitored over a 4-wk period using an ELISA system for soluble $p24^{gag}$ expression (DuPont, NEN, Inc., Billerica, MA). CAT activity was measured 48 h after infection after repeated (four) cell washes. As a negative control, virus was heat inactivated by incubation at 56°C for 60 min.

IL-2 Secretion. IL-2 gene expression was stimulated by the addition of PMA (40 nM) in EL4 cells or PMA (40 nM), and PHA 1 μg/ml) in Jurkat cells. Supernatant was collected 24 h after stimulation. Secreted IL-2 in culture supernatants was measured with the IL-2-dependent CTLL cell line and a colorimetric assay (see Fig. 4). Cell proliferation expressed as OD_{570} is on the ordinate. The concentration of supernatant in uninduced (−) or stimulated (+) EL4 or Jurkat cells was 10%. Values represent the average of triplicate determinations, and SDs were <10%.

Results

An amphotropic retroviral vector that encodes the M10 mutant of Rev, as well as a control vector expressing β-galactosidase, were used to stably transduce the leukemic T cell lines CEM, EL-4, and Jurkat. To analyze the effects of constitutive Rev M10 expression in long-term cell culture, subclones derived from the CEM-transduced cells were established. Immunoprecipitation analysis with an anti-Rev antiserum confirmed that subclones 3, 4, and 5, but not the control cells, expressed the transdominant Rev protein (data not shown). Continued expression of the cell surface receptor CD4 was confirmed by FACS® analysis (data not shown). These cell lines were next tested for their ability to support

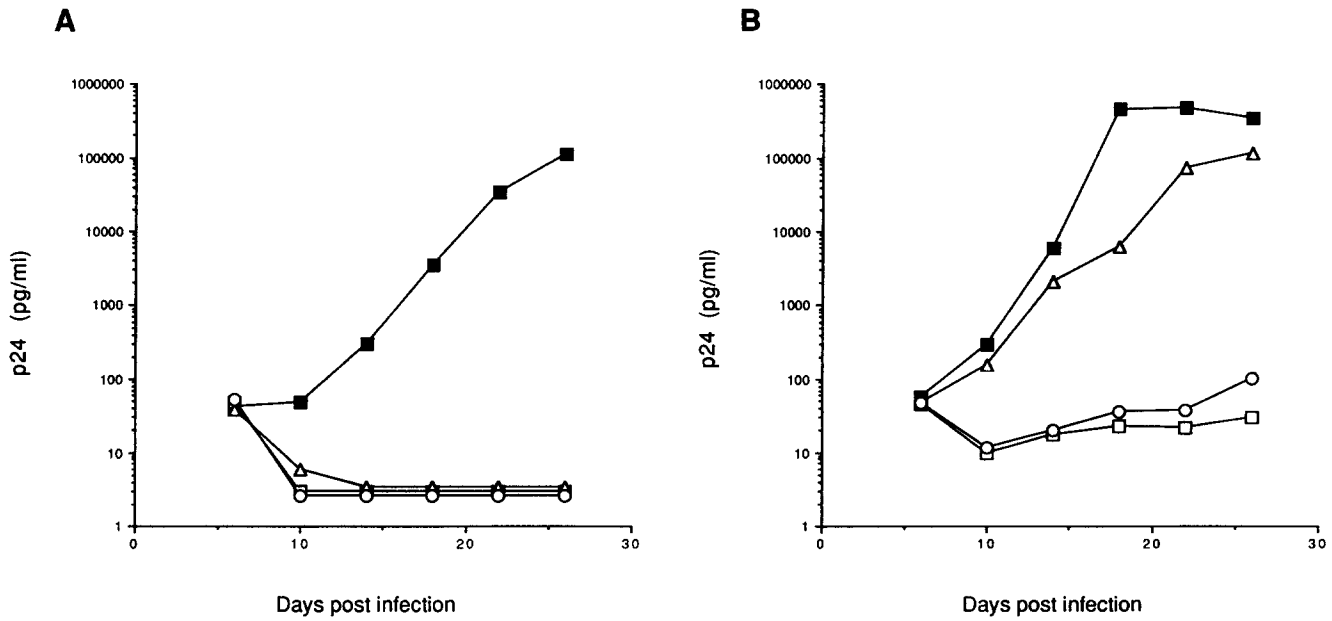

Figure 1. The inhibitory effect of Rev M10 protein on the replication of cloned (*A*) and uncloned (*B*) HIV-1 in CEM cells. The PLJ-M10 and PLJ-BAG retroviral vectors (20) were used to stably transduce CEM cells (Materials and Methods). After subcloning, 5×10^5 cells expressing BAG (■) or Rev M10 (□) subclone 3; (△) subclone 4; (○) subclone 5 were infected. Results are representative of two (*A*) or four (*B*) independent experiments.

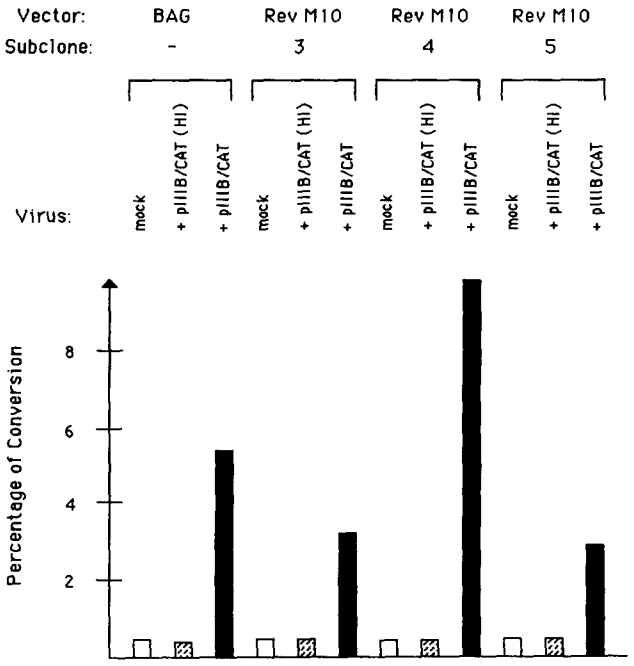

Figure 2. Susceptibility of CEM cells to initial infection by HIV-1. The indicated CEM subclones (5×10^5 cells) were either mock infected with media alone, infected with heat-inactivated (HI) virus, or infected with virus derived from pIIIB/CAT. Cells were maintained as described in the legend to Fig. 1, washed at 48 h, and assayed for CAT activity. Results are representative of two independent experiments.

a productive infection of HIV-1. All three Rev M10–expressing subclones were highly resistant to productive infection with virus generated from a molecular clone (Fig. 1 *A*) (23). In contrast, cells transduced with the β-galactosidase vector were similar in susceptibility to nontransduced CEM cells (Fig. 1 *A*, and data not shown). When infected with the heterogeneous HTLV-IIIB pool (24), two of the M10-expressing CEM subclones displayed resistance to highly productive infection (subclones 3 and 5, Fig. 1 *B*). The continuous production of low, but detectable levels of virus by these cultures suggested that these subclones were infected by HIV, but that virus failed to replicate efficiently in these cells. In contrast, subclone 4 displayed only a slight delay in the pattern of viral spread relative to control cells. We have recently reconfirmed the validity of transdominant Rev-mediated protection of cells against HIV infection using an alternative transdominent mutant of Rev, M32 (11), CEM-SS cells (25), and a double-copy retroviral vector, N2A (26) (data not shown).

If the observed resistance of the CEM cells to productive HIV-1 infection was a direct consequence of Rev M10 expression, then the cells should still be susceptible to initial infection. To address this issue, a proviral expression vector pIIIB/CAT was constructed in which the viral *nef* gene was replaced with the bacterial CAT gene. Because the Nef protein is expressed early after HIV-1 infection and is therefore independent of Rev (3), it is predicted that, in cells infected with pIIIB/CAT virus, the induction of CAT activity shortly after exposure to virus would be a measure of early viral gene expression and, hence, the number of infectious events. As anticipated, all the CEM lines expressed readily detectable CAT activity 48 h after infection with the pIIIB/CAT virus (Fig. 2). No increase in CAT activity was detected in cells infected with heat-inactivated (HI) virus relative to mock-infected cells, thus demonstrating that detection of CAT was a consequence of retroviral infection and not residual inoculum.

To evaluate the effect of Rev M10 on T cell function, the CEM subclones were transfected with HIV-CAT reporter plasmids. Mitogen and cytokine induction of the HIV LTR is dependent on an 11-bp sequence recognized by the NF-κB transcription factor (27). To determine whether the sensitivity of the HIV LTR to NF-κB was affected by the M10 mutant, CAT activity in each CEM subclone was determined after PMA induction. Activation mediated by the κB site was indistinguishable between the three subclones of CEM M10, compared with the β-galactosidase control (Fig. 3 *A*). In addition, stimulation by the HIV-1 Tat protein, through the TAR region of the HIV-CAT reporter, was also unaffected in subclones that stably express M10 protein (Fig. 3 *B*). To

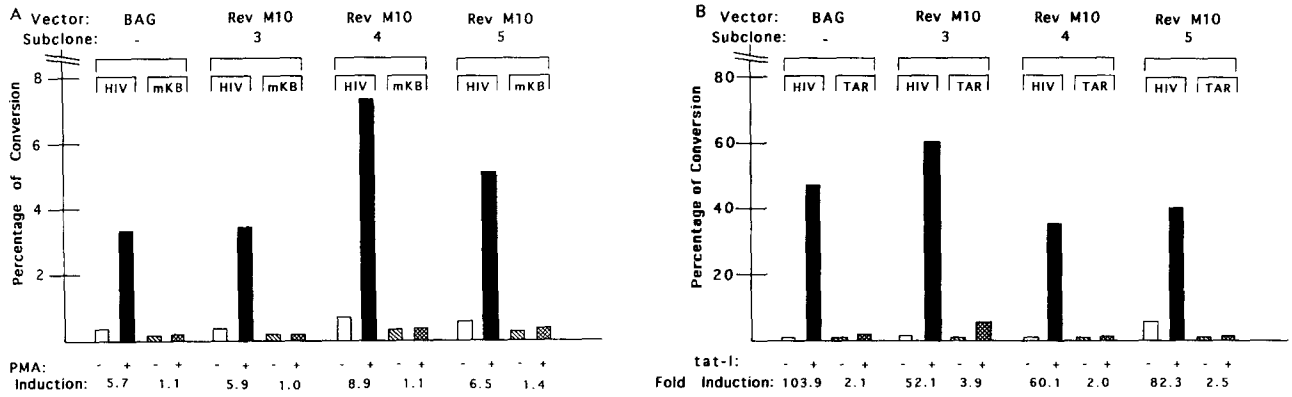

Figure 3. Stimulation of HIV-1 transcription by NF-κB (*A*) or Tat (*B*) in CEM cells that stably express the Rev M10 protein. To analyze NF-κB inducibility (*A*), the indicated CEM subclones were transfected with 10 μg of HIV-CAT plasmid or a mutant κB HIV-CAT plasmid before PMA stimulation. To analyze Tat function (*B*), these subclones were cotransfected with HIV-CAT or with a derivative bearing a defective TAR element (29). Cells were untreated (−) or incubated (+) with PMA (20 ng/ml), and CAT assays were performed as previously described (27).

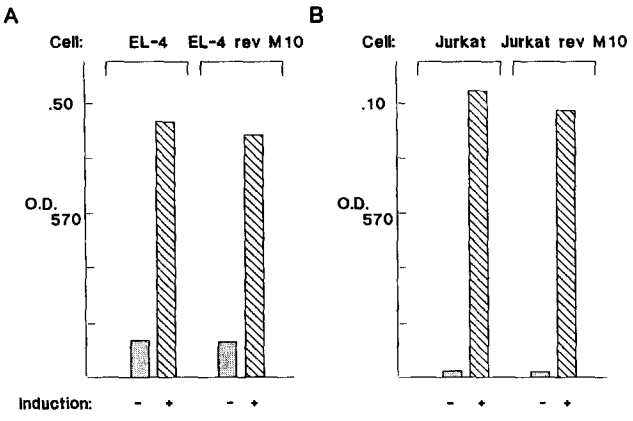

Figure 4. Induction of IL-2 secretion after stimulation in (*A*) EL-4 cells and (*B*) Jurkat cells that express the Rev M10 protein.

examine more thoroughly the effect of constitutive M10 expression on the immune function of cells, transduced Jurkat and EL-4 cell lines were evaluated for their ability to secrete IL-2 after mitogen activation. No difference was observed with respect to IL-2 secretion in either cell compared to controls (Fig. 4), thus demonstrating that induction of this lymphokine in cells that stably expressed the Rev transdominant protein is unaffected.

Discussion

The introduction into susceptible cells of mutant forms of HIV-1 proteins that then interfere with viral replication represents an attractive approach to the treatment of HIV-1 infection (1, 2). Although analogues of HIV-1 nucleic acid structures, such as TAR or RRE, have been used to inhibit HIV replication in cells (28), the potential of mutant viral proteins to achieve this effect, and their ability to interfere with normal cellular function, remain unknown. Here we demonstrate a substantial inhibition of HIV-1 replication in the majority of T cells that constitutively express the Rev M10 transdominant mutant. In addition, we show that the transdominant Rev-expressing lines vary in their initial susceptibility to HIV-1 infection by no more than twofold relative to the control, BAG-expressing, cells (Fig. 2). These data support the conclusion, therefore, that resistance of the CEM cells to HIV-1 infection is directly due to the M10 protein, which inhibits productive infection by preventing expression of viral structural proteins. The mechanism of Rev M10 protection has been analyzed previously (17). The RNA binding and multimerization domains of M10 are intact, suggesting that the Rev M10 multimerizes and precludes the binding of wild-type Rev to the RRE and subsequent interactions with the cellular factors that facilitate the cytoplasmic expression of unspliced viral RNA (17). We speculate that subclone 4 displayed only a delay in the onset of acute infection with HTLV-IIIB (Fig. 1 *B*), because it appears to show increased susceptibility to initial infection, as determined by the CAT infection experiment (Fig. 2), relative to the other lines.

Importantly, expression of Rev M10 protein did not interfere with the induction of several T cell activation genes, including the NF-κB transcription factor, those required for Tat function, and the multiple regulatory proteins that control IL-2 synthesis. Although the possibility remains that Rev M10 may affect other activation pathways, these results suggest that stable expression of Rev M10 does not adversely affect T cell function globally. The Rev M10 protein therefore represents a gene product that might preclude efficient HIV-1 replication without adversely affecting T cell function. Although differential protection by Rev M10 was observed in separate subclones with the uncloned virus pool, resistance need not be conferred equally on all T cells to provide a therapeutic effect. For example, T cell clones rendered resistant to HIV-1 could proliferate to reconstitute the immune system of an infected host. Because multiple cells can be transduced, protection against HIV-1-induced disease might be achieved by transducing sufficiently large numbers of cells with Rev M10. This gene could be readily introduced into peripheral $CD4^+$ T cells or eventually into progenitor cells that give rise to mature $CD4^+$ populations. This strategy might, therefore, be used to slow the progression of AIDS in asymptomatic HIV-1-infected patients or, possibly, might be used to restore immune function in individuals that have progressed to later disease stages.

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