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Induction of CD4 Expression and Human Immunodeficiency Virus Type 1 Replication by Mutants of the Interferon-Inducible Protein Kinase PKR

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Replication of the human immunodeficiency virus type 1 (HIV-1) is inhibited by interferons (IFNs), and the IFN-inducible protein kinase PKR is thought to mediate this effect by regulating protein synthesis. Here we report that ectopic expression of dominant negative PKR mutants in Jurkat cells induces HIV-1 replication. Specifically, expression of CD4 is upregulated by the PKR mutants, and this correlates with an induction of HIV-1 binding and proviral DNA synthesis upon HIV-1 infection. Moreover, activation of NF-κB was induced by an RNA binding-defective mutant of PKR. Thus, it appears that PKR, in addition to translational control, is involved in HIV-1 replication by modulating virus binding through the regulation of CD4 expression and virus gene expression through the activation of NF-κB.

Interferons (IFNs) inhibit human immunodeficiency virus type 1 (HIV-1) replication in both chronically and acutely infected cells (reviewed in reference 44) by blocking both early and late stages of HIV-1 replication (9, 39, 45, 52–54). For example, treatment with IFNs results in a dramatic decrease in proviral DNA accumulation and interferes with the initiation of HIV-1 reverse transcription (RT) (1, 52, 53). Furthermore, IFNs inhibit HIV-1 replication due to specific inhibition of mRNA translation (9) and can block the assembly and release of virions in promonocytic cells, T cells chronically infected by HIV-1, and peripheral blood T cells acutely infected with the virus (15, 21, 43, 45).

In response to IFNs, a large number of genes are induced. This process can be attributed both to specific responsive elements in the promoter regions of IFN-responsive genes and to the presence and activation of specific *trans*-activation factors (reviewed in reference 51). Among the different IFN-induced proteins, PKR is a serine/threonine protein kinase which is activated by autophosphorylation upon binding to double-stranded RNA (dsRNA) (reviewed in reference 46). Activated PKR then phosphorylates the α subunit of eukaryotic translation initiation factor 2, a modification that causes inhibition of protein synthesis (22). In addition to its antiviral function (24, 38), PKR exhibits an antiproliferative function (8, 27) and a tumor suppressor function in vitro (4, 27, 36).

PKR is thought to play an important role in HIV-1 infection. For example, the HIV-1 transactivator-responsive RNA (TAR) has been shown to bind to and modify PKR activity in vitro due to its extensive secondary structure (14, 19, 20, 32, 48). To prevent the decrease in viral protein synthesis, HIV-1 has evolved strategies to overcome PKR activation. For instance, productive HIV-1 infection has been shown to be accompanied by a reduction of cellular levels of PKR, which is mediated by the HIV-1 Tat protein (49). Interestingly, PKR and Tat can associate in vitro and in vivo, and this interaction is suggested to be one of the mechanisms by which HIV-1 bypasses the antiviral action of IFNs (34). In addition to translational control, a recent report shows that PKR is involved in the transcriptional activation of HIV-1 5′ long terminal repeat (5′ LTR) (37).

We further investigated the role of PKR in HIV-1 infection by examining the regulation of HIV-1 replication in Jurkat cells transfected with mutants of PKR. The PKR mutants were either the dominant negative catalytically inactive PKRΔ6 (deletion of the 6 amino acids Leu^{361}-Phe-Ile-Gln-Met-Glu^{366} within catalytic subdomain V, which results in a protein that lacks kinase activity but is still able to bind dsRNA [27]) or the dsRNA binding-defective mutant PKRLS9 (mutations of Ala^{66} Ala^{68} to Gly^{66} Pro^{68}, which completely abolishes binding to dsRNA [18]). Here we show that expression of either of the PKR mutants results in an induction of HIV-1 replication. Specifically, expression of CD4, the major receptor of HIV-1 (11, 25), is induced by both PKR mutants at the transcriptional level with PKRLS9 exhibiting the strongest effect. Upregulation of CD4 by PKRLS9 correlated with an increase in HIV-1 binding and proviral DNA synthesis following HIV-1 infection.

Generation and characterization of Jurkat cells expressing the PKR mutants. Ten micrograms of wild-type (wt) PKR (35), PKRΔ6 (27), or PKRLS9 (18) cDNA under the control of the human cytomegalovirus (CMV) promoter in pcDNAI/neo vector (Invitrogen) was electroporated into Jurkat cells (10^7; ATCC TIB-152) at 250 V and 960 μF (Bio-Rad). Selection of cells in G418 (800 μg/ml) and isolation of clones were performed as previously described (26). Expression of transfected genes was first identified by an RT-PCR method (Fig. 1a). Briefly, RNA (1 μg) treated with RNase-free DNase I was reverse transcribed with, as a 3′ primer, an oligonucleotide (5′-CCAAATCCACCTGAGCCAATTA-3′) which hybridizes

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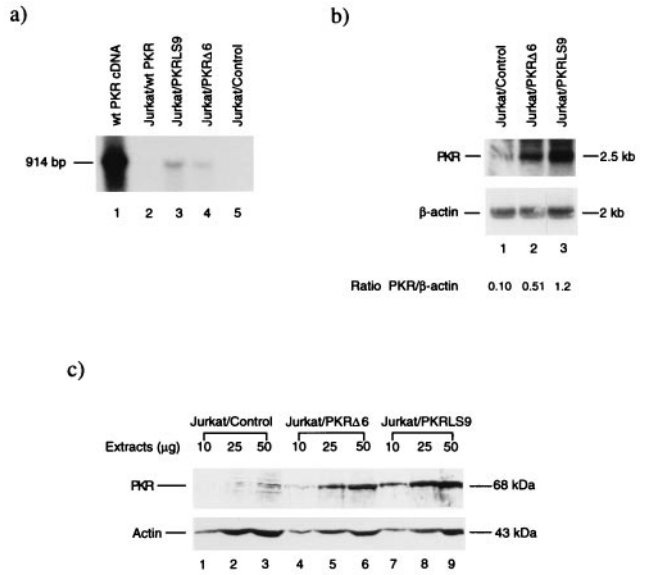

FIG. 1. Characterization of Jurkat cells expressing mutants of PKR. (a) Transfection of Jurkat cells and detection of PKR RNA expression by RT-PCR. The detection of exogenous PKR cDNA expression was performed by RT-PCR as described in the text. RT-PCR products (914 bp) were analyzed on 2% agarose gel by electrophoresis and subjected to Southern blot analysis with a ^{32}P-labeled human PKR cDNA probe. Human PKR cDNA amplified by PCR was used as a positive control (lane 1). Other lanes show Jurkat cells transfected with wt PKR cDNA (lane 2), PKRLS9 cDNA (lane 3), PKRΔ6 cDNA (lane 4), or pcDNAI/neo DNA (lane 5; negative control). (b) Detection of PKR RNA by Northern blot analysis. Total RNA (10 μg) was subjected to Northern blot analysis (50) using either ^{32}P-labeled human PKR cDNA (upper panel) or ^{32}P-labeled β-actin cDNA as a probe (lower panel). Lane 1, Jurkat/Control cells (expressing the neomycin resistance gene only); lane 2, Jurkat/PKRΔ6 cells; lane 3, Jurkat/PKRLS9 cells. The ratios of PKR to β-actin RNA are indicated. (c) Immunoblotting analysis of PKR. The preparation of cell extracts and immunoblot analysis of PKR were performed as described elsewhere (26, 27). Lanes 1 to 3, cytoplasmic extracts from Jurkat/Control cells; lanes 4 to 6, Jurkat/PKRΔ6 cells; lanes 7 to 9, Jurkat/PKRLS9 cells. Immunoblot analysis of actin (lower panel) was used to normalize the levels of PKR protein.

1.0 kb downstream of the first AUG in the PKR mRNA. The synthesized cDNAs were amplified at 30 cycles (94°C for 1 min, 64°C for 1.5 min, and 72°C for 2 min) with the above 3′ primer and a 5′ primer (5′-CACTGCTTAACTGGCTTATCGA-3′), which is complementary to a sequence located within the CMV promoter downstream of the transcription initiation site and therefore is specific for exogenous PKR cDNAs. The PCR products were subjected to Southern blot analysis (50) to detect the levels of amplified PKR cDNAs. In three separate transfection experiments, detection of exogenous wt PKR RNA in stable transfectants by RT-PCR was not possible (Fig. 1a, lane 2), most probably due to cell growth inhibition mediated by wt PKR overexpression (8, 27). In this regard, wt PKR can be overexpressed in some transformed cell lines without apparent inhibition of cell growth (26) possibly due to modulation of its activity by specific inhibitors (23, 30, 40).

In contrast to wt PKR, expression of PKRLS9 and PKRΔ6 was readily detected by RT-PCR (Fig. 1a, lanes 3 and 4, respectively) and was then verified by Northern blot and immunoblot analyses. Northern blot analysis (Fig. 1b) showed a 5- and 12-fold increase of PKR RNA in cells transfected with PKRΔ6 (lane 2) and PKRLS9 cDNA (lane 3), respectively, relative to endogenous PKR (lane 1). At the protein level, immunoblot analysis with a mouse antibody specific to human PKR (Fig. 1c) that recognizes the carboxyl terminus of the protein (3) showed an approximately three- and fivefold increase of PKR protein in cells expressing PKRΔ6 (lanes 4 to 6) and PKRLS9 (lanes 7 to 9), respectively, compared to endogenous PKR protein levels (lanes 1 to 3). Similar data were obtained with several other Jurkat clones expressing either of the PKR mutants (data not shown).

Induction of HIV-1 replication by the PKR mutants. Jurkat cells expressing the neomycin resistance gene only (herein referred to as Jurkat/Control cells) and Jurkat cells expressing either PKRLS9 (Jurkat/PKRLS9) or PKRΔ6 (Jurkat/PKRΔ6) were then infected with HIV-1 (Fig. 2). A low multiplicity of infection (MOI) (Fig. 2b, MOI of 0.001) was also used to mimic physiological conditions. The production of HIV-1 was monitored by quantifying the amount of HIV-1 reverse transcriptase in the culture supernatant every other day after infection (29). HIV-1 replication was induced in both Jurkat/PKRLS9 and Jurkat/PKRΔ6 cells compared to Jurkat/Control cells (Fig. 2a and b). However, HIV-1 replication was higher and faster in Jurkat/PKRLS9 than Jurkat/PKRΔ6 cells. Eighty percent of the cells remained viable until 15 days after infection as determined by trypan blue dye exclusion, indicating that expression of either of the PKR mutants does not induce any

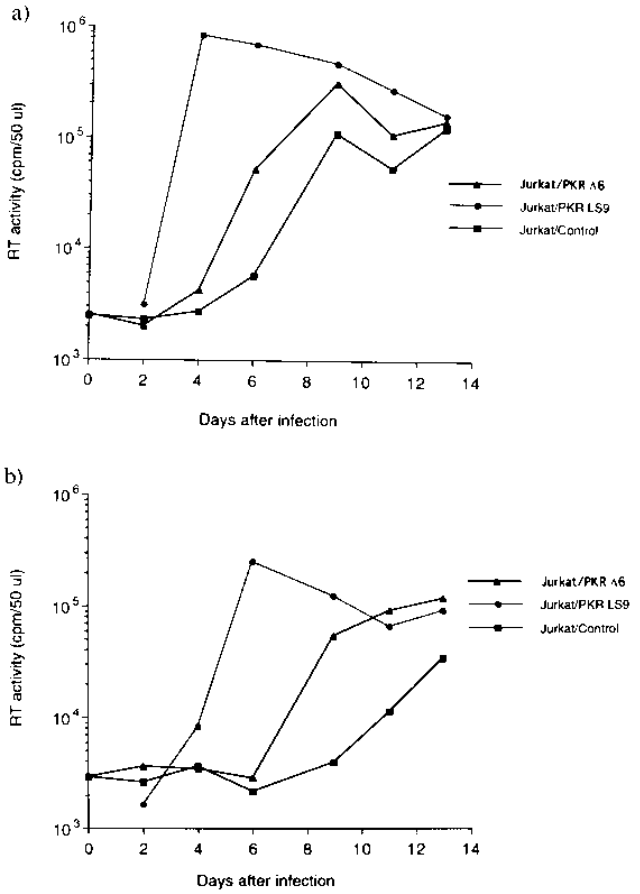

FIG. 2. Induction of HIV-1 replication in Jurkat cells expressing PKR mutants. Jurkat cells (2×10^6) were infected with the HXB2D molecular clone of HIV-1 (16) in serum-free medium for 2 h at MOIs of 0.1 (a) and 0.001 (b). Cells were then grown in complete medium for 2 weeks. Cell supernatants were collected every second day and analyzed for virus reverse transcriptase (RT) activity as previously described (29). Reverse transcriptase levels for Jurkat/Control (closed squares), Jurkat/PKRΔ6 (closed triangles), or Jurkat/PKRLS9 (closed circles) cells are indicated. Each value represents the average of triplicates in which the difference was less than 10%. This is one of five reproducible experiments.

cytopathic effect of Jurkat cells. It is noteworthy that the progeny virus was capable of reinfecting and replicating in Jurkat cells, indicating that expression of either PKR mutant did not affect virus infectivity (data not shown). Similar results were obtained with other Jurkat/PKRΔ6 and Jurkat/PKRLS9 clones (data not shown).

Induction of CD4 expression in Jurkat cells expressing the mutants of PKR. The induction of HIV-1 replication by the PKR mutants in Jurkat cells could be exerted at any of the several stages of the virus life cycle, such as virus entry (binding to CD4 and fusion), RT, DNA integration, gene expression, assembly, and budding.

HIV-1 binding was examined by incubating an equal number of Jurkat cells with an equal amount of virus (MOI, 0.5) for 5 min at room temperature and removing unbound HIV-1 by extensive washing. Total RNA was then isolated and reverse transcribed with the M661 primer (5′-CCTGCGTCGAGAG AGCTCCTCTGG-3′ [55]). The resulting cDNA was subjected to 25 cycles of amplification by PCR (denaturation at 91°C for 1 min and polymerization at 65°C for 2 min) using the M661 and M667 (5′-GGCTAACTAGGGAACCCACTG-3′ [55]) set of primers. PCR products were quantified by Southern blotting using the LTR/gag DNA region of HIV-1 cDNA as a probe. As shown in Fig. 3a, the amount of cell-bound HIV-1 was higher for Jurkat/PKRLS9 (lanes 2 to 4) or Jurkat/PKRΔ6 (lanes 5 to 7) than for Jurkat/Control cells (lanes 8 to 10). However, Jurkat/PKRLS9 cells exhibited significantly higher levels of HIV-1 binding than Jurkat/PKRΔ6 cells (Fig. 3a, compare lanes 2 to 4 with 5 to 7). These data suggested that expression of PKR mutants facilitates virus binding most likely by upregulating the expression of one or more receptor molecules. This possibility was further examined by quantifying the levels of CD4, the major HIV-1 receptor (11, 25). Immunostaining of CD4 and fluorescence-activated cell sorter (FACS) analysis revealed that the surface expression of CD4 was induced ~10-fold in Jurkat/PKRLS9 cells and ~3-fold in Jurkat/PKRΔ6 cells, respectively, relative to Jurkat/Control cells (Fig. 3b). These results correlate well with the amount of cell-bound HIV-1 and suggest that upregulation of CD4 is most probably the reason for induced virus binding.

To examine the molecular mechanism(s) of CD4 expression by PKR mutants, we then analyzed CD4 RNA expression by Northern blotting (Fig. 3c). The levels of CD4 RNA were increased ~15-fold in Jurkat/PKRLS9 cells (lanes 11 to 15) and ~4-fold in Jurkat/PKRΔ6 cells (lanes 6 to 10) compared to Jurkat/Control cells (lanes 1 to 5). Since regulation of CD4 expression occurs at the level of transcription and/or RNA stability (7, 31), we then examined whether CD4 RNA stability was affected by either of the PKR mutants. Following actinomycin D treatment, the RNA levels of CD4 (upper panel) or β-actin (lower panel) remained stable in all cells, showing that neither of the PKR mutants affects CD4 RNA stability. In the same experiment, the levels of c-myc RNA were decreased in all cells (middle panel), showing that actinomycin D was effective. Similar data were obtained with several Jurkat/PKRΔ6 and Jurkat/PKRLS9 clones (data not shown). These findings favor a transcriptional regulation of CD4 by the PKR mutants in Jurkat cells.

Induction of proviral DNA synthesis by PKRLS9. The upregulation of CD4 by the PKR mutants may account, at least in part, for the induction of HIV-1 replication (Fig. 2) by facilitating early steps of the virus life cycle such as virus entry and proviral DNA formation. This possibility was further investigated by quantifying the levels of proviral DNA in cells expressing PKRLS9 or PKRΔ6. To this end, we employed a semiquantitative PCR method, which measures the rates of

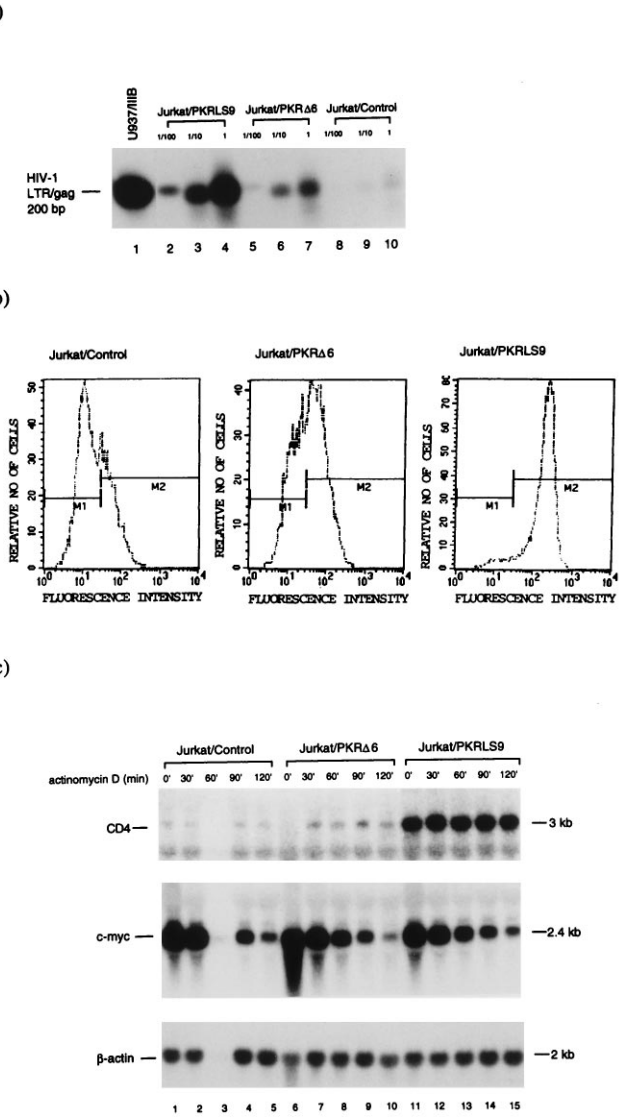

FIG. 3. Induction of CD4 expression by the PKR mutants. (a) Induction of HIV-1 binding to cells expressing mutants of PKR. Quantification of cell-bound HIV-1 was performed by RT-PCR as described in the text. After amplification, PCR products were subjected to 2% agarose electrophoresis and quantified by Southern blotting using a ^{32}P-labeled fragment of LTR/gag cDNA as a probe. As a positive control, RNA from chronically infected U937 cells (U937/IIIB) was used in lane 1. Lanes 2 to 4, Jurkat/PKRLS9 cells; lanes 5 to 7, Jurkat/PKRΔ6 cells; lanes 8 to 10, Jurkat/Control cells. Tenfold (lanes 3, 6, and 9) and 100-fold (lanes 2, 5, and 8) dilutions of reverse transcribed products were used to show the linearity of PCR amplification. (b) Induction of surface CD4 expression by the PKR mutants. Jurkat/Control, Jurkat/PKRΔ6, and Jurkat/PKRLS9 cells (10^6) were subjected to surface immunostaining by incubation with a mouse anti-CD4 monoclonal antibody (anti-OKT4; 1 μg/ml in PBS plus 0.5% bovine serum albumin) followed by incubation with fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin G (BioCan; 1 μg/ml in PBS plus 0.5% bovine serum albumin). Cells were then analyzed on a cytofluorograph (FACStar; Becton Dickinson, Mountain View, Calif.) as previously described (26). The marker M1 indicates fluorescence intensity of cells stained with the secondary fluorescein isothiocyanate-conjugated antibody only (negative control), whereas M2 indicates the CD4-positive population. (c) Induction of CD4 RNA expression by mutants of PKR. Expression of CD4 mRNA was determined by Northern blot analysis using ^{32}P-labeled human CD4 cDNA as a probe (upper panel). The CD4 RNA stability was examined by treating cells with actinomycin D (10 μg/ml) for 30 (lanes 2, 7, and 12), 60 (lanes 3, 8, and 13), 90 (lanes 4, 9, and 14), or 120 (lanes 5, 10, and 15) min prior to RNA extraction. As a control to actinomycin D, Northern blots were reprobed with ^{32}P-human c-myc cDNA (middle panel). Expression of CD4 mRNA was normalized to the levels of β-actin mRNA (lower panel). Lanes 1 to 5, Jurkat/Control cells; lanes 6 to 10, Jurkat/PKRΔ6 cells; lanes 11 to 15, Jurkat/PKRLS9 cells.

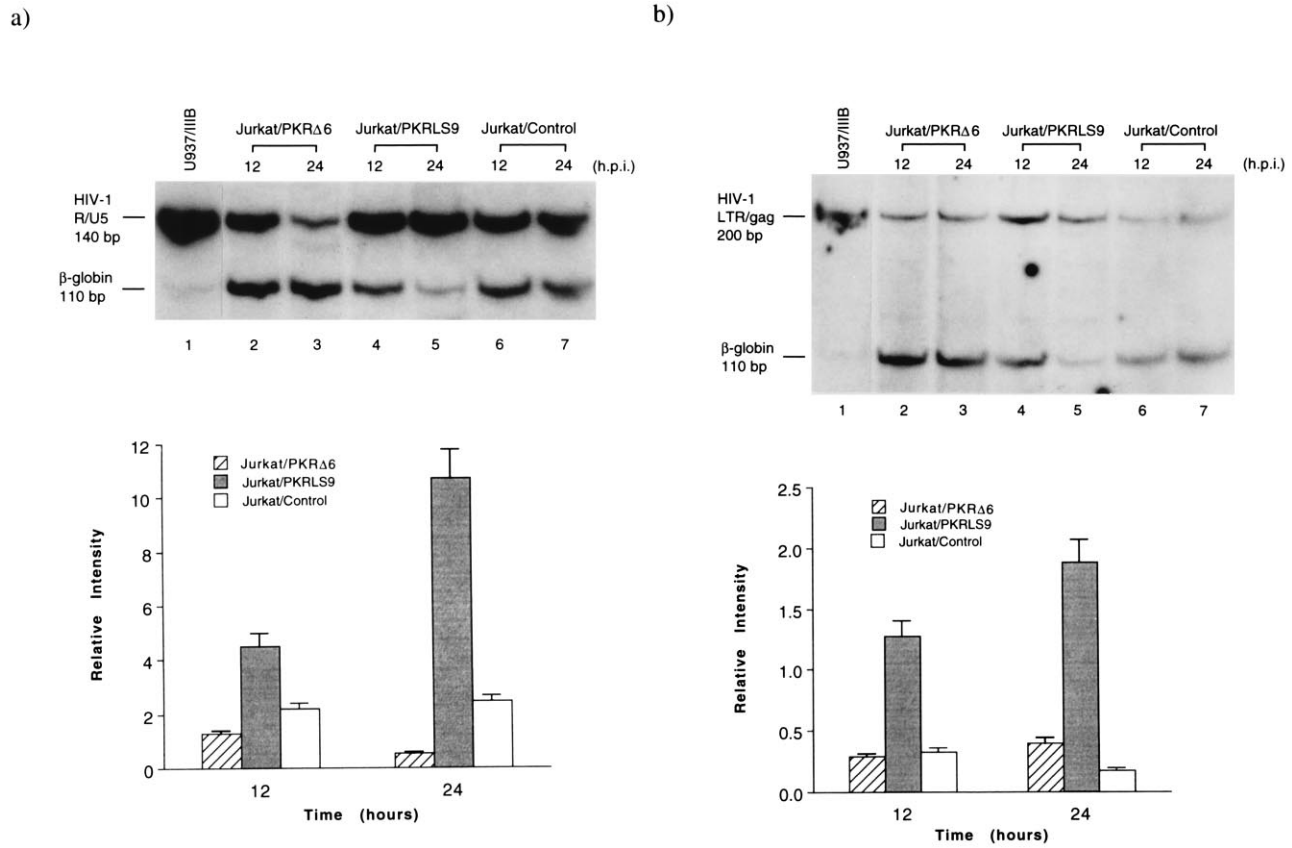

FIG. 4. Induction of HIV-1 proviral DNA synthesis in Jurkat/PKRLS9 cells. The formation of proviral DNA at the initial (a) or final (b) step of RT in Jurkat cells infected with HIV-1 (MOI, 0.05) for 12 (lanes 2, 4, and 6) or 24 (lanes 3, 5, and 7) h was measured by the semiquantitative PCR method established by Zack et al. (55). As a positive control, DNA from chronically infected U937 cells (U937/IIIB) was used in lane 1. Lanes 2 and 3, Jurkat/PKRΔ6 cells; lanes 4 and 5, Jurkat/PKRLS9 cells; lanes 6 and 7, Jurkat/Control cells. (Lower panel) Quantification of radioactive bands of HIV-1 and β-globin cDNAs was performed by a laser densitometer (Ultroscan XL; LKB). The relative intensity was calculated after normalization with β-globin cDNA. Each value represents the average of triplicates in which the difference was less than 10%.

proviral DNA formation at the initial or the final step of virus RT (55). Accordingly, Jurkat cells (5×10^6) were infected with HIV-1 at an MOI of 0.05 and cellular DNA was extracted and isolated 12 or 24 h postinfection (55). The initial (early) step of RT (Fig. 4a) was detected with an oligonucleotide primer pair specific for the R/U5 region of the LTR (M667/AA55, 5′-CTGCTAGAGATTTTCCACACTGAC-3′) which flanks sequences within the first region of the viral DNA synthesized during RT. The final step of RT (Fig. 4b) was analyzed by PCR using the HIV-1 oligonucleotide primer pair M667/M661 (LTR/gag primers), which flanks the tRNA primer binding site (PBS) of the HIV-1 genome and detects full-length or nearly completely synthesized viral DNA. Moreover, a primer pair specific for human β-globin sequences (5′ primer, 5′-ACACA ACTGTGTTCACTAGC-3′; 3′ primer, 5′-CAACTTCATCC ACGTTCACC-3′) within the first exon of the gene was included in each reaction. PCR amplification of sample DNA (2 μg) was performed in the presence of 0.2 ng of the ^{32}P-end-labeled 5′ primer at 25 cycles (denaturation at 91°C for 1 min and polymerization at 65°C for 2 min), and PCR products were analyzed by electrophoresis on 8% nondenaturing polyacrylamide gels and autoradiography.

This and two other experiments revealed a three- to fivefold increase in the initial step (Fig. 4a, lower panel) and a four- to sixfold increase in the final step (Fig. 4b, lower panel) of RT in Jurkat/PKRLS9 cells. In Jurkat/PKRΔ6 cells, however, no significant effect on proviral DNA synthesis was observed. These data suggest that expression of PKRLS9 causes an induction of proviral DNA synthesis most probably as a result of CD4 upregulation. The lack of a measurable induction of proviral DNA synthesis in Jurkat/PKRΔ6 cells may be due to the lower levels of CD4 expression compared to Jurkat/PKRLS9 cells (Fig. 3).

Expression of PKRLS9 antagonizes CD4 RNA downmodulation upon HIV-1 infection. CD4 plays a critical role in the establishment of a productive HIV-1 infection. A loss in CD4 expression in vitro occurs several days postinfection, when sufficient progeny virions are produced (7). The best-characterized mechanism is the retention and degradation of newly synthesized CD4 in the endoplasmic reticulum (7). However, some reports have suggested that both transcriptional and posttranslational mechanisms are implicated in CD4 downmodulation (7).

We were interested in investigating how the induction of CD4 expression by the PKR mutants after HIV-1 infection was brought about. To this end, we analyzed the CD4 RNA by Northern blotting 5, 10, and 15 days postinfection. The levels of CD4 RNA were stable in Jurkat/Control, Jurkat/PKRΔ6, and Jurkat/PKRLS9 cells until 10 days after infection (Fig. 5a). However, at day 15 a rapid downmodulation of CD4 RNA occurred in Jurkat/Control cells (lane 12) and Jurkat/PKRΔ6 cells (lane 8). In contrast, CD4 RNA levels remained stable

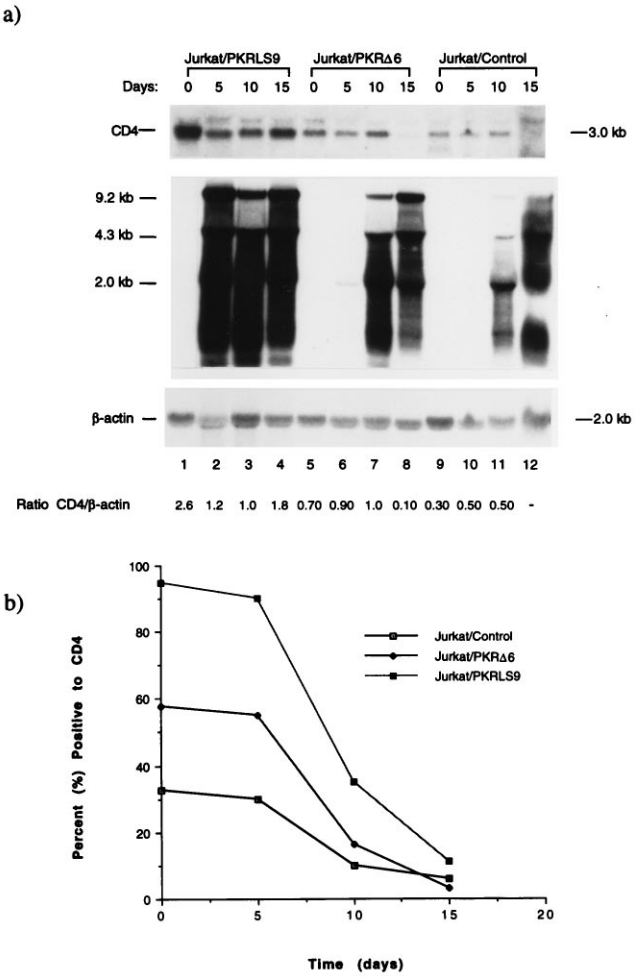

FIG. 5. PKRLS9 antagonizes CD4 mRNA downmodulation during HIV-1 replication. (a) CD4 RNA expression in Jurkat cells expressing the mutants of PKR following HIV-1 infection. Jurkat/PKRLS9 (lanes 1 to 4), Jurkat/PKRΔ6 (lanes 5 to 8), and Jurkat/Control (lanes 9 to 12) cells were infected with equal amounts of HIV-1 (MOI, 0.05). Total RNA (10 μg) was isolated 5, 10, and 15 days postinfection and subjected to Northern blot analysis. Blots were probed with ^{32}P-labeled human CD4 cDNA (upper panel), ^{32}P-labeled LTR/gag region of HIV-1 cDNA (middle panel), or the ^{32}P-labeled entire cDNA of β-actin (lower panel). The 9.2-, 4.3-, and 3.0-kb RNA species of HIV-1 and the ratios of CD4 to β-actin RNA are indicated. This is one of three reproducible experiments. (b) The kinetics of surface CD4 expression in Jurkat cells expressing the PKR mutants. Cells were incubated with anti-human CD4 (OKT4) monoclonal antibody and fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin G followed by analysis on a FACStar cytofluorograph. Infected cells were fixed in 1% paraformaldehyde in PBS prior to FACS analysis. A minimum of 2×10^4 cells were analyzed. This is one of two reproducible experiments.

in Jurkat/PKRLS9 cells infected with HIV-1 (lane 4). The amount of HIV-1 RNAs (middle panel) was higher in Jurkat/PKRLS9 cells than in Jurkat/PKRΔ6 or Jurkat/Control cells, and their expression was detected as early as 5 days postinfection (lane 2). These results suggest that expression of PKRLS9 antagonizes the downmodulation of CD4 RNA during HIV-1 infection.

Next, we examined the levels of CD4 protein expression on the cell surface by immunostaining and FACS analysis. CD4 protein was downmodulated in all cell lines at days 10 and 15 postinfection (Fig. 5b). Although the levels of surface CD4 expression per se were higher in Jurkat/PKRLS9 cells than in Jurkat/PKRΔ6 and Jurkat/Control cells, the rate of CD4 downmodulation was similar in all cell types. These data suggest that PKRLS9 controls the downmodulation of CD4 RNA but not CD4 protein in HIV-1-infected Jurkat cells.

Induction of NF-κB DNA binding by PKRLS9. HIV-1 infection may utilize a physiological pathway(s) to control gene expression (7). The HIV-1 5′ LTR contains a κB binding site and requires NF-κB activation for efficient viral gene expression (12, 47). Interestingly, PKR has been implicated in NF-κB activation (28, 33), probably through the phosphorylation of IκBα (28). Therefore, expression of the PKR mutants may modulate NF-κB activation in HIV-1-infected Jurkat cells.

Activation of NF-κB was examined in Jurkat/Control, Jurkat/PKRΔ6, and Jurkat/PKRLS9 cells after treatment with phorbol 12-myristate 13-acetate (PMA), which has been suggested to mimic signaling cascades induced by binding of HIV gp120 to CD4 (7, 47). The ability of activated NF-κB to bind DNA was measured by electrophoretic mobility shift assays using a ^{32}P-labeled double-stranded DNA (dsDNA) oligonucleotide bearing the κB binding site of HIV-1 5′ LTR and nuclear extracts from untreated or PMA-treated cells. As shown in Fig. 6a, PMA treatment induced the formation of four major κB dsDNA-protein complexes (A, B, C, and D) in all cells (lanes 2, 4, and 6), which were competed by an excess of cold dsDNA oligonucleotide (lane 7). However, formation of complexes A, B, and C was more highly induced in Jurkat/PKRLS9 (lane 4) than in Jurkat/PKRΔ6 (lane 6) or Jurkat/Control (lane 2) cells. To examine the subunit composition of κB-binding complexes, we performed gel supershift assays by incubating nuclear extracts from PMA-treated Jurkat/Control or Jurkat/PKRLS9 cells with antisera against p65, rel, or p50 protein. As shown in Fig. 6b, complexes A and B were eliminated by antisera against p50 (lanes 2 and 7) or p65 (lanes 3 and 8), whereas complex C was eliminated by antisera against p50 (lanes 2 and 7) or c-rel (lanes 4 and 9). Formation of the supershifted DNA-protein complexes was detected after long exposures (data not shown). Most likely then, complexes A and B consist of p50/p65 heterodimers whereas complex C consists of p50/c-rel heterodimers. Interestingly, complex D was not affected by any of the above antisera, indicating that most likely this complex is not NF-κB. These data show that expression of PKRLS9 but not of PKRΔ6 facilitates the induction of NF-κB DNA binding in PMA-treated Jurkat cells.

To further substantiate the activation of NF-κB by PKRLS9, the chloramphenicol acetyltransferase (CAT) reporter gene under the control of the wt HIV-1 LTR (ptzIIICAT [6]) or a mutant form of LTR lacking the κB site (ptzIIICAT −109/−79 [6]) was transfected into Jurkat/Control, Jurkat/PKRΔ6, and Jurkat/PKRLS9 cells. After PMA treatment, CAT expression driven by the wt but not by mutant HIV-1 LTR was induced ~2.5-fold in Jurkat/PKRLS9 cells compared to either Jurkat/Control or Jurkat/PKRΔ6 cells (Fig. 6c). These data are in line with the levels of NF-κB DNA binding induced by the PKR mutants and show a stimulatory effect of PKRLS9 on both NF-κB DNA binding and transactivation.

CD4 plays a critical role in T-cell development, T-cell activation, and regulation of the immune response (31). However, the mechanism(s) of regulation of CD4 expression by PKR is not immediately clear. Experiments with actinomycin D favor a transcriptional regulation, which may be exerted at the level of either initiation or elongation. At the level of initiation, it is possible that PKR regulates translation of a transcriptional factor(s) involved in CD4 expression. In this regard, PKRΔ6 and mutants of PKR defective in RNA binding similar to PKRLS9 were shown to function as transdominant inhibitors of PKR in phosphorylation of eukaryotic initiation factor 2α in vivo and regulation of protein synthesis (2, 4, 13). The higher

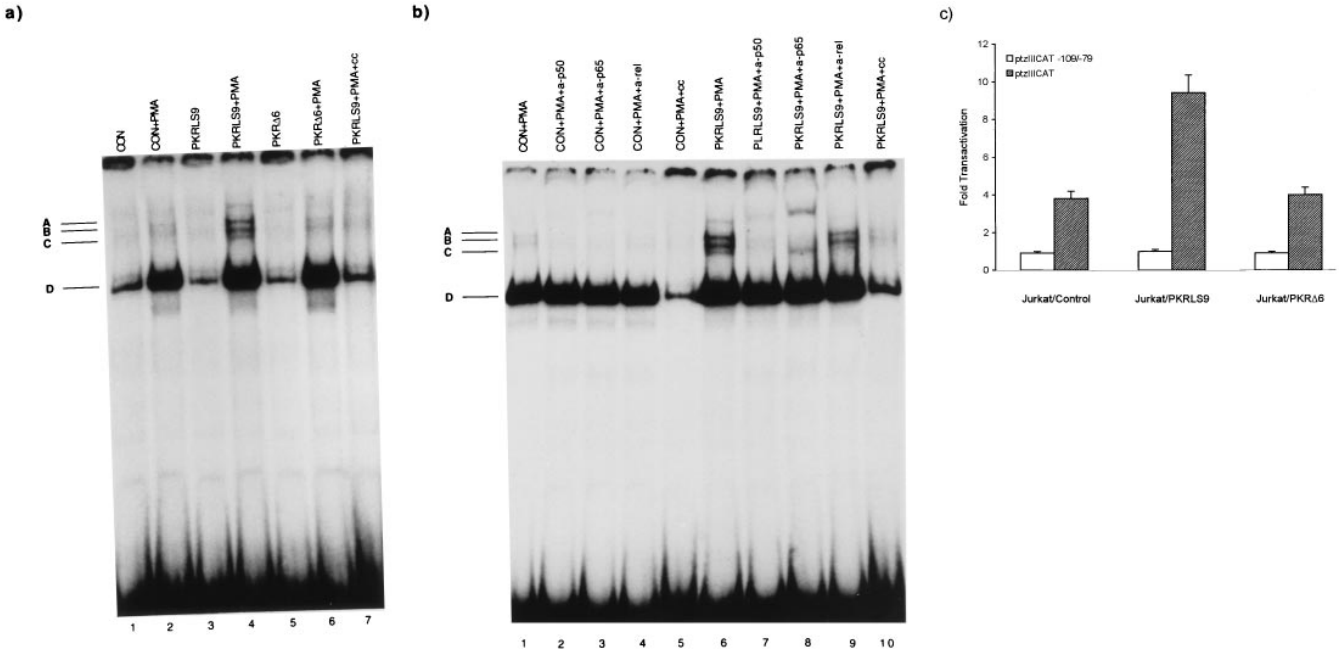

FIG. 6. Regulation of NF-κB activity by the PKR mutants. (a) Induction of NF-κB DNA binding in Jurkat/PKRLS9 cells in response to PMA. Nuclear extracts (5 μg) from Jurkat/Control (lanes 1 and 2), Jurkat/PKRLS9 (lanes 3, 4, and 7), or Jurkat/PKRΔ6 (lanes 5 and 6) cells before (lanes 1, 3, and 5) or after (lanes 2, 4, 6, and 7) PMA treatment (50 ng/ml, 6 h) were analyzed for NF-κB activity by binding to 80 pg of a 5′-end-^{32}P-labeled HIV-κB dsDNA oligonucleotide (5×10^6 cpm/ng; 5′-AGCTGGGACTTTCCGCTA-3′; the underlined sequence corresponds to the NF-κB binding site) as previously described (26). For cold competition, a 125-fold excess of unlabeled HIV-1 κB dsDNA oligonucleotide was added in nuclear extracts from PMA-treated PKRLS9 cells (lane 7). (b) Analysis of NF-κB subunit composition by antibody supershift assays. Nuclear extracts (10 μg) from PMA-treated Jurkat/Control (lanes 1 to 5) or Jurkat/PKRLS9 (lanes 6 to 10) cells were incubated with specific antisera to p50 (lanes 2 and 7), p65 (lanes 3 and 8), or c-rel (lanes 4 and 9) and tested for NF-κB binding to the ^{32}P-labeled HIV-1 κB dsDNA oligonucleotide. Lanes 5 and 10, cold competition. (c) Induction of NF-κB transactivation by PKRLS9. Jurkat/Control, Jurkat/PKRΔ6, and Jurkat/PKRLS9 cells were transiently transfected with 12.5 μg of either ptzIIICAT or ptzIIICAT −109/−79 vector together with 2.5 μg of CMV–β-galactosidase plasmid as described elsewhere (6). PMA (50 ng/ml) was added 32 h after transfection, and cells were incubated for an additional 12 h. CAT activity was assayed in whole-cell extracts (50) and normalized to levels of β-galactosidase. Fold transactivation for each sample represents the ratio of CAT activity after PMA treatment to CAT activity without PMA treatment.

levels of CD4 in Jurkat/PKRLS9 cells than in Jurkat/PKRΔ6 cells may be explained by a stronger translational effect mediated by PKRLS9 due to its higher levels of expression than PKRΔ6 (Fig. 1b and c). The regulation of CD4 expression adds to several previous reports for a transcriptional role of PKR (26, 28, 33, 41). In addition to transcription initiation, the inhibition of normal attenuation through the pausing of CD4 transcription might be another explanation for generating the elevated amounts of CD4 RNA.

Upregulation of CD4 is most probably the reason for the increased levels of virus binding and proviral DNA synthesis in Jurkat/PKRLS9 cells after HIV-1 infection. However, the possibility that PKRLS9 also induces the expression of another protein required for virus binding and entry, such as the newly identified HIV-1 coreceptor fusin (5), cannot be excluded. HIV-1 uses multiple mechanisms to control CD4 expression consistent with CD4 being an important regulator of viral replication and pathogenesis (7). Our findings show that PKRLS9 but not PKRΔ6 prevents the downmodulation of CD4 RNA in HIV-1-infected cells, indicating that the RNA binding rather than the catalytic properties of PKR is important in this process. HIV-1 infection perhaps induces interaction(s) of PKR with viral and/or cellular components through its RNA-binding domain, which modifies the structure and/or function of PKR, resulting in a transcriptional regulation of CD4. Such interactions may be mediated by Tat and TAR viral products (34, 48) and/or the cellular TAR RNA-binding protein (17), which has been shown to bind PKR (10) and modify its function (42). Removal of cell surface CD4 is apparently necessary and sufficient to block superinfection by both HIV-1 and HIV-2 (7), and therefore, downmodulation of CD4 by PKR might be one of the mechanisms to prevent superinfection.

Our data show that PKRLS9 can induce the activation of NF-κB, and this may also be responsible for the high levels of HIV-1 replication in Jurkat/PKRLS9 cells. Considering the ability of PKR to phosphorylate IκBα in vitro (28), the induction of NF-κB DNA binding and transactivation in Jurkat/PKRLS9 cells implies phosphorylation of IκBα by PKRLS9 since this mutant of PKR is catalytically intact. However, this would not be compatible with a dominant negative function of PKRLS9 as shown for RNA binding-defective mutants of PKR (2, 4). In addition, consistent with our previous report (26), NF-κB activation is not inhibited by the dominant negative PKRΔ6. One possible explanation, then, for these paradoxical data is that PKRLS9 modulates the expression of a protein(s) (kinase or phosphatase) involved in IκBα phosphorylation to a higher degree than does PKRΔ6. A further conceivable explanation may be that PKRLS9 but not PKRΔ6 modulates a pathway(s) of NF-κB activation that is downstream of IκBα phosphorylation.

In conclusion, we show that dominant negative mutants of PKR induce CD4 expression, and this is an important mechanism for HIV-1 replication in vitro. The regulation of CD4 expression by PKR may have important implications not only in HIV-1 infection in vivo but also in many physiological events that utilize CD4 (31). Further experiments are required to elucidate the molecular mechanisms of CD4 transcription by

PKR and its implication in CD4-mediated signaling and immune response.

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REFERENCES

1. **Baca-Regen, L., N. Heinzinger, M. Stevenson, and H. E. Gendelman.** 1995. Alpha interferon-induced antiretroviral activities: restriction of viral nucleic acid synthesis and progeny virion production in human immunodeficiency virus type 1-infected monocytes. J. Virol. **68:**7559–7565.
2. **Barber, G. N., R. Jagus, E. F. Meurs, A. G. Hovanessian, and M. G. Katze.** 1995. Molecular mechanisms responsible for malignant transformation by regulatory and catalytic domain variants of the interferon-induced enzyme RNA-dependent protein kinase. J. Biol. Chem. **270:**17423–17428.
3. **Barber, G. N., M. G. Katze, and A. Darveau.** Personal communication.
4. **Barber, G. N., M. Wambach, S. Thompson, R. Jagus, and M. G. Katze.** 1995. Mutants of the RNA-dependent protein kinase (PKR) lacking double-stranded RNA binding domain I can act as transdominant inhibitors and induce malignant transformation. Mol. Cell. Biol. **15:**3138–3146.
5. **Bates, P.** 1996. Chemokine receptors and HIV-1: an attractive pair? Cell **86:**1–3.
6. **Beauraplant, P., H. Kwon, M. Clarke, R. Lin, N. Sonenberg, M. Wainberg, and J. Hiscott.** 1996. Transdominant mutants of IκBα block Tat-Tumor necrosis factor synergistic activation of human immunodeficiency virus type 1 gene expression and virus multiplication. J. Virol. **70:**5777–5785.
7. **Bour, S., R. Geleziunas, and M. A. Wainberg.** 1995. The human immunodeficiency virus type 1 (HIV-1) CD4 receptor and its central role in promotion of HIV-1 infection. Microbiol. Rev. **59:**63–93.
8. **Chong, K. L., L. Feng, K. Schappert, E. Meurs, T. F. Donahue, J. D. Friesen, A. G. Hovanessian, and B. R. G. Williams.** 1992. Human p68 kinase exhibits growth suppression in yeast and homology to the translational regulator GCN2. EMBO J. **11:**1553–1562.
9. **Coccia, E. M., B. Krust, and A. G. Hovanessian.** 1994. Specific inhibition of viral protein synthesis in HIV infected cells in response to interferon treatment. J. Biol. Chem. **269:**23087–23094.
10. **Cosentino, G. P., S. Venkatesan, F. C. Serluca, S. R. Green, M. B. Mathews, and N. Sonenberg.** 1995. Double-stranded-RNA-dependent protein kinase and TAR RNA-binding protein form homo- and heterodimers *in vivo*. Proc. Natl. Acad. Sci. USA **92:**9445–9449.
11. **Dalgleish, A. G., P. C. L. Beverly, P. R. Clapman, D. H. Crawford, M. F. Greaves, and R. A. Weiss.** 1984. The CD4 (T4) antigen is an essential component of the receptor for AIDS retrovirus. Nature (London) **312:**763–767.
12. **DeLuca, C., A. Roulston, A. Koromilas, M. A. Wainberg, and J. Hiscott.** 1996. Chronic human immunodeficiency virus type 1 infection of myeloid cells disrupts the autoregulatory control of the NF-κB/Rel pathway via enhanced IκBα degradation. J. Virol. **70:**5183–5193.
13. **Donze, O., R. Jagus, A. E. Koromilas, J. W. B. Hershey, and N. Sonenberg.** 1995. Abrogation of translation initiation factor eIF-2 phosphorylation causes malignant transformation of NIH3T3 cells. EMBO J. **14:**3828–3834.
14. **Edery, I., R. Petryshyn, and N. Sonenberg.** 1989. Activation of double-stranded RNA-dependent kinase (dsI) by the TAR region of HIV-1 mRNA: a novel translational control mechanism. Cell **56:**303–312.
15. **Fernie, B. F., G. Poli, and A. S. Fauci.** 1991. Alpha interferon suppresses virion but not soluble human immunodeficiency virus antigen production in chronically infected T-lymphocytic cells. J. Virol. **65:**3968–3971.
16. **Fisher, A. G., E. Collalti, L. Ratner, R. C. Gallo, and F. Wong-Staal.** 1985. A molecular clone of HTLV-III with biological activity. Nature (London) **316:**262–265.
17. **Gatignol, A., A. Buckler-White, B. Berkhout, and K.-T. Jeang.** 1991. Characterization of a human TAR RNA-binding protein that activates the HIV-1 LTR. Science **251:**1597–1600.
18. **Green, S. R., and M. B. Mathews.** 1992. Two RNA-binding motifs in the double-stranded RNA-activated protein kinase, DAI. Genes Dev. **6:**2478–2490.
19. **Gunnery, S., S. R. Green, and M. B. Mathews.** 1992. Tat-responsive region RNA of human immunodeficiency virus type 1 stimulates protein synthesis *in vivo* and *in vitro*: relationship between structure and function. Proc. Natl. Acad. Sci. USA **89:**11557–11561.
20. **Gunnery, S., A. P. Rice, H. D. Robertson, and M. B. Mathews.** 1990. Tat-responsive region RNA of human immunodeficiency virus 1 can prevent activation of the double-stranded-RNA-activated protein kinase. Proc. Natl. Acad. Sci. USA **87:**8687–8691.
21. **Hansen, B. D., P. L. Nara, R. K. Maheshwari, G. S. Sidhu, J. G. Bernbaum, D. Hoekzema, M. S. Meltzer, and H. E. Gendelman.** 1995. Loss of infectivity by progeny virus from alpha interferon-treated human immunodeficiency virus type 1-infected T cells is associated with defective assembly of envelope gp120. J. Virol. **66:**7543–7548.
22. **Hershey, J. W. B.** 1989. Protein phosphorylation controls translation rates. J. Biol. Chem. **264:**20823–20826.
23. **Judware, R., and R. Petryshyn.** 1992. Mechanism of action of a cellular inhibitor of the dsRNA-dependent protein kinase from 3T3-F442A cells. J. Biol. Chem. **267:**21685–21690.
24. **Katze, M. G.** 1995. Regulation of the interferon-induced PKR: can viruses cope? Trends Microbiol. **3:**75–78.
25. **Klatzmann, D., E. Champagne, S. Chamaret, J. Gruest, D. Guetard, T. Hercend, J.-C. Gluckman, and L. Montagnier.** 1984. T-lymphocyte T4 molecule behaves as the receptor for human retrovirus LAV. Nature (London) **312:**767–768.
26. **Koromilas, A. E., C. Cantin, A. W. B. Craig, R. Jagus, J. Hiscott, and N. Sonenberg.** 1995. The interferon-inducible protein kinase PKR modulates the transcriptional activation of immunoglobulin κ gene. J. Biol. Chem. **270:**25426–25434.
27. **Koromilas, A. E., S. Roy, G. N. Barber, M. G. Katze, and N. Sonenberg.** 1992. Malignant transformation by a mutant of the IFN-inducible dsRNA-dependent protein kinase. Science **257:**1685–1689.
28. **Kumar, A., J. Haque, J. Lacoste, J. Hiscott, and B. R. G. Williams.** 1994. Double-stranded RNA-dependent protein kinase activates transcription factor NF-κB by phosphorylating IκB. Proc. Natl. Acad. Sci. USA **91:**6288–6292.
29. **Lee, M. H., K. Sano, F. E. Morales, and D. T. Imagawa.** 1988. Comparable sensitivities for detection of human immunodeficiency virus by sensitive reverse transcriptase and antigen capture enzyme-linked immunosorbent assays. J. Clin. Microbiol. **26:**371–374.
30. **Lee, T. G., N. Tang, J. Miller, and M. G. Katze.** 1994. The 58,000-dalton cellular inhibitor of the interferon-induced double-stranded RNA-activated protein kinase (PKR) is a member of the tetratricopeptide repeat family of proteins. Mol. Cell. Biol. **14:**2331–2342.
31. **Littman, D. R. (ed.).** 1996. The CD4 molecule: roles in T lymphocytes and in HIV disease. Curr. Top. Microbiol. Immunol. **205:**1–182.
32. **Maitra, R. K., N. A. J. McMillan, A. Desai, J. McSwiggen, A. G. Hovanessian, G. Sen, B. R. G. Williams, and R. H. Silverman.** 1994. HIV-1 TAR RNA has an intrinsic ability to activate interferon-inducible enzymes. Virology **204:**823–827.
33. **Maran, A., R. K. Maitra, A. Kumar, B. Dong, W. Xiao, G. Li, B. R. G. Williams, P. F. Torrence, and R. H. Silverman.** 1994. Blockage of NF-κB signaling by selective ablation of an mRNA target by 2-5A antisense chimeras. Science **265:**789–792.
34. **McMillan, N. A. J., R. F. Chun, D. P. Siderovski, J. Galabru, W. M. Toone, C. E. Samuel, T. W. Mak, A. G. Hovanessian, K.-T. Jeang, and B. R. G. Williams.** 1995. HIV-1 Tat directly interacts with the interferon-induced, double-stranded RNA-dependent kinase, PKR. Virology **213:**413–424.
35. **Meurs, E., K. Chong, J. Galabru, N. Shaun, B. Thomas, I. M. Kerr, B. R. G. Williams, and A. G. Hovanessian.** 1990. Molecular cloning and characterization of the human double-stranded RNA-activated protein kinase induced by interferon. Cell **62:**379–390.
36. **Meurs, E. F., J. Galabru, G. N. Barber, M. G. Katze, and A. G. Hovanessian.** 1993. Tumor suppressor function of the interferon-induced double-stranded RNA-activated protein kinase. Proc. Natl. Acad. Sci. USA **90:**232–236.
37. **Meurs, E. F., N. McMillan, B. R. G. Williams, A. G. Hovanessian, and P. J. Southern.** 1995. Human PKR transfected into murine cells stimulates expression of genes under the control of the HIV-1 or HTLV-1 LTR. Virology **214:**653–659.
38. **Meurs, E. F., Y. Watanabe, S. Kadereit, G. N. Barber, M. G. Katze, K. Chong, B. R. G. Williams, and A. G. Hovanessian.** 1992. Constitutive expression of human double-stranded RNA-activated p68 kinase in murine cells mediates phosphorylation of eukaryotic initiation factor 2 and partial resistance to encephalomyocarditis virus growth. J. Virol. **66:**5805–5814.
39. **Meylan, P. R. A., J. C. Guatelli, J. R. Munis, D. D. Richman, and R. S. Kornbluth.** 1993. Mechanisms for the inhibition of HIV replication by interferons-alpha, -beta, and gamma in primary human macrophages. Virology **193:**138–148.
40. **Mundschau, L. J., and D. V. Faller.** 1992. Oncogenic *ras* induces an inhibitor of double-stranded RNA-dependent eukaryotic initiation factor 2α-kinase activation. J. Biol. Chem. **267:**23092–23098.

41. **Offermann, M. K., J. Zimring, K. H. Mellits, M. K. Hagan, R. Shaw, R. M. Medford, M. B. Mathews, S. Goodbourn, and R. Jagus.** 1995. Activation of the double-stranded-RNA-activated protein kinase and induction of vascular cell adhesion molecule-1 by poly(I) poly(C) in endothelial cells. Eur. J. Biochem. **232:**28–36.
42. **Park, H., M. V. Davies, J. O. Langland, H.-W. Chang, Y. S. Nam, J. Tartaglia, E. Paoletti, B. L. Jacobs, R. J. Kaufman, and S. Venkatesan.** 1994. TAR RNA-binding protein is an inhibitor of the interferon-induced protein kinase PKR. Proc. Natl. Acad. Sci. USA **91:**4713–4717.
43. **Perno, C.-F., A. Bergamini, C. D. Pesce, G. Milanese, M. Capozzi, S. Aquaro, S. Thaisrivongs, W. G. Tarpley, G. Zon, C. D'Agostini, G. Rocchi, E. Garaci, and R. Calio.** 1993. Inhibition of the protease of human immunodeficiency virus blocks replication and infectivity of the virus in chronically infected macrophages. J. Infect. Dis. **168:**1148–1156.
44. **Pitha, P. M.** 1994. Multiple effects of interferon on the replication of human immunodeficiency virus type 1. Antivir. Res. **24:**205–219.
45. **Poli, G., J. M. Orenstein, A. Kinter, T. M. Folks, and A. S. Fauci.** 1989. Interferon-alpha but not AZT suppresses HIV expression in chronically infected cell lines. Science **244:**575–577.
46. **Proud, C. G.** 1995. PKR: a new name and new roles. Trends Biochem. Sci. **20:**241–246.
47. **Roulston, A., R. Lin, P. Beauparlant, M. A. Wainberg, and J. Hiscott.** 1995. Regulation of human immunodeficiency virus type 1 and cytokine gene expression in myeloid cells by NF-κB/Rel transcription factors. Microbiol. Rev. **59:**481–505.
48. **Roy, S., M. B. Agy, A. G. Hovanessian, N. Sonenberg, and M. G. Katze.** 1991. The integrity of the stem structure of human immunodeficiency virus type 1 Tat-responsive sequence RNA is required for interaction with the interferon-induced 68,000-M_r protein kinase. J. Virol. **65:**632–640.
49. **Roy, S., M. G. Katze, N. Parkin, I. Edery, A. G. Hovanessian, and N. Sonenberg.** 1990. Control of the interferon-induced 68-kilodalton protein kinase by HIV-1 tat gene product. Science **247:**1216–1219.
50. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
51. **Schindler, C., and J. E. Darnell.** 1995. Transcriptional responses to polypeptide ligands: the JAK-STAT pathway. Annu. Rev. Biochem. **64:**621–651.
52. **Shirazi, Y., and P. M. Pitha.** 1993. Interferon alpha-mediated inhibition of human immunodeficiency virus type 1 provirus synthesis in T cells. Virology **193:**303–312.
53. **Shirazi, Y., and P. M. Pitha.** 1995. Alpha interferon inhibits early stages of the human immunodeficiency virus type 1 replication cycle. J. Virol. **66:** 1321–1328.
54. **Smith, M. S., R. J. Thresher, and J. S. Pagano.** 1991. Inhibition of human immunodeficiency virus type 1 morphogenesis in T cells by alpha interferon. Antimicrob. Agents Chemother. **35:**62–67.
55. **Zack, J. A., S. J. Arrigo, S. R. Weitsman, A. S. Go, A. Haislip, and I. S. Y. Chen.** 1990. HIV-1 entry into quiescent primary lymphocytes: molecular analysis reveals a labile, latent viral structure. Cell **61:**213–222.