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Chronic Human Immunodeficiency Virus Type 1 Infection of Myeloid Cells Disrupts the Autoregulatory Control of the NF-κB/Rel Pathway via Enhanced IκBα Degradation

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Productive human immunodeficiency virus type 1 (HIV-1) infection causes sustained NF-κB DNA-binding activity in chronically infected monocytic cells. A direct temporal correlation exists between HIV infection and the appearance of NF-κB DNA-binding activity in myelomonoblastic PLB-985 cells. To examine the molecular basis of constitutive NF-κB DNA-binding activity in HIV-1-infected cells, we analyzed the phosphorylation and turnover of IκBα protein, the activity of the double-stranded RNA-dependent protein kinase (PKR) and the intracellular levels of NF-κB subunits in the PLB-985 and U937 myeloid cell models. HIV-1 infection resulted in constitutive, low-level expression of type 1 interferon (IFN) at the mRNA level. Constitutive PKR activity was also detected in HIV-1-infected cells as a result of low-level IFN production, since the addition of anti-IFN-α/β antibody to the cells decreased PKR expression. Furthermore, the analysis of IκBα turnover demonstrated an increased degradation of IκBα in HIV-1-infected cells that may account for the constitutive DNA binding activity. A dramatic increase in the intracellular levels of NF-κB subunits c-Rel and NF-κB2 p100 and a moderate increase in NF-κB2 p52 and RelA(p65) were detected in HIV-1-infected cells, whereas NF-κB1 p105/p50 levels were not altered relative to the levels in uninfected cells. We suggest that HIV-1 infection of myeloid cells induces IFN production and PKR activity, which in turn contribute to enhanced IκBα phosphorylation and subsequent degradation. Nuclear translocation of NF-κB subunits may ultimately increase the intracellular pool of NF-κB/IκBα by an autoregulatory mechanism. Enhanced turnover of IκBα and the accumulation of NF-κB/Rel proteins may contribute to the chronically activated state of HIV-1-infected cells.

$CD4^+$ myeloid cells are important targets for human immunodeficiency virus (HIV) infection and are susceptible to infection at multiple stages of differentiation (38, 53). HIV infection of mononuclear phagocytes in vivo occurs predominantly in terminally differentiated macrophages in tissues such as the lymph nodes, lung, skin, and brain (23), although HIV-1-infected myeloid precursors in the bone marrow have been identified in AIDS patients (46). Since HIV is not cytopathic to myeloid cells, these cell populations may act as reservoirs of HIV within the bone marrow and other tissues, facilitating virus spread and accelerating disease progression (33). Physiological changes associated with HIV infection of myeloid cells include functional alterations in chemotaxis, phagocytosis, lytic activity, antigen presentation, and cytokine gene expression (reviewed in references 23 and 53). Generally, constitutive cytokine mRNA expression is not observed in chronically HIV-infected THP-1, PLB-985, and U937 cell models. Nonetheless, increases in cytokine mRNA levels are seen after cellular stimulation by inducers such as lipopolysaccharide or Sendai virus (13, 14, 37, 63). HIV infection of the promonocytic U937 cells induced phenotypic changes associated with cellular differentiation, leading to increased virus expression (19, 29, 40, 41, 45). Previously, we demonstrated that chronic HIV infection of PLB-985 cells leads to morphological characteristics and expression of myeloid-specific surface markers (CD14 and c-*fms*) consistent with irreversible differentiation along the monocytic pathway (52). The expression of surface receptors associated with differentiation and activation may lead to increased responsiveness to bacteria and cytokines secreted by neighboring cells.

Chronic HIV infection of myeloid cells may also alter signaling pathways that modulate the host inflammatory response. One of the important transcriptional pathways involves the NF-κB/Rel proteins, a family of ubiquitous transcription factors that participates in various biological processes including immunological responsiveness, lymphoid differentiation, and cell growth regulation (reviewed in reference 53). NF-κB/Rel proteins are present in most cell types in an inactive cytoplasmic form, complexed to inhibitory IκB proteins. NF-κB DNA-binding activity can be induced following exposure to a wide variety of activating agents including cytokines, T-cell mitogens, bacterial products, viruses, and double-stranded RNA (dsRNA) (3, 4, 17). Activation of NF-κB requires the disruption of IκB-DNA-binding subunit interactions, which is mediated by phosphorylation of IκB followed by proteolytic degradation via the ubiquitin-proteasome pathway (1, 12, 15, 39, 61).

The cloning of several NF-κB genes showed that these proteins share a region of homology, termed the NF-κB/Rel/dorsal region, which spans approximately 300 amino acids at the amino terminus of each protein. Two of the DNA-binding subunits are synthesized as inactive cytoplasmic precursor proteins. The product of the *nfkb1* gene is a 105-kDa protein which gives rise to a 50-kDa DNA-binding subunit, p50 (10, 25, 36). The *nfkb2*-encoded p100 precursor is very similar to p105,

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and its mature DNA-binding form is p52. The other NF-κB/Rel DNA-binding proteins are not processed from precursors: c-Rel is an 85-kDa protein, which in addition to the NF-κB/Rel/dorsal region, contains a unique 300-amino-acid carboxy terminal domain that is related to RelA(p65) (11, 24) encoded by the *rela* gene. The carboxy termini of c-Rel and RelA contain two activation domains similar to VP16 (5, 20, 44, 59). RelB is also structurally similar to RelA and c-Rel but lacks *trans*-activating potential and has an elongated amino-terminal segment (57).

The IκB family of proteins is defined by its ability to interact with NF-κB/Rel subunits, sequestering them in the cytoplasm, possibly in association with cytoskeletal proteins (9). The IκB proteins so far identified contain between five and seven repeats of a 33-amino-acid sequence termed the ankyrin motif. The nuclear localization signal in the NF-κB/Rel/dorsal domain of the DNA-binding subunit is necessary for this interaction and is masked by IκB in the complex (8, 21). Members of the IκB family include, IκBα, IκBβ, IκBγ, the proto-oncogene *bcl-3*, and the precursors p105 and p100 (reviewed in reference 7).

We and others have previously demonstrated that productive virus infection causes sustained NF-κB DNA-binding activity in chronically HIV-infected monocytic cell lines (2, 42, 47, 50, 52, 60). A direct temporal correlation exists between HIV infection and the appearance of NF-κB DNA-binding activity in the myelomonoblastic PLB-985 cells (51, 52). One common point at which the infection cycle of many viruses may overlap is with the generation of dsRNA during replication which may in turn activate the dsRNA-dependent kinase (PKR) (34, 58). PKR is induced by interferon (IFN) and is activated by binding to dsRNA molecules synthesized during viral infection. In addition to a well-characterized effect on host translation, PKR is implicated in the activation of NF-κB DNA-binding activity in vitro and in vivo. PKR has been shown to phosphorylate IκBα and activate NF-κB DNA-binding activity in cellular extracts (27). Furthermore, NF-κB DNA-binding activity is not activated by dsRNA [poly (rI):poly (rC)] in HeLa cells depleted of PKR by a novel antisense method (30).

In order to examine the molecular basis of constitutive NF-κB DNA-binding activity, we analyzed the expression of individual NF-κB subunits, IκBα turnover, and PKR expression in the PLB-985 and U937 cell models. Our results demonstrated increased intracellular levels of NF-κB subunits c-Rel and NF-κB2 and increased turnover of IκBα in HIV-1-infected cells. We also identified in HIV-1-infected cells increased levels of PKR protein and activity which was elevated by constitutive low-level IFN production. The addition of anti-IFN-α/β antibody to the cells decreased PKR by 48 h after the addition. We suggest that HIV-1 infection of myeloid cells induces IFN production and PKR activity, which in turn contributes to enhanced IκBα phosphorylation and subsequent degradation. Nuclear translocation of NF-κB subunits may ultimately increase the intracellular pool of NF-κB/IκBα by an autoregulatory mechanism. The increased pool of NF-κB/IκBα may contribute to the chronically activated state of HIV-1-infected cells.

MATERIALS AND METHODS

Cell culture. Myelomonoblastic PLB-985 and U937 cells and PLB-IIIB and U9-IIIB cells, infected with HIV strain IIIB, were maintained in RPMI 1640 (GIBCO, Life Technologies Inc., Grand Island, N.Y.) supplemented with 5% Fetal Clone (Hyclone, Logan, Utah), 2 mM L-glutamine, and 10 μg of gentamicin (Schering Canada, Pointe Claire, Quebec, Canada) per ml. The cells were stimulated with either 50 ng of phorbol myristate acetate (PMA; Sigma) per ml, 10 μg tumor necrosis factor alpha (TNF-α; R&D Systems) 1,000 IU of IFNα (Schering Canada) per ml or 1,000 new units (NU) of anti-leukocyte IFN antibody (anti-IFN-α/β) (Sigma) per ml in the medium. Cells were seeded at 5×10^5 cells per ml and stimulated for different times depending on the experiment.

S1 nuclease analysis. Total cellular RNA from PLB-985 and PLB-IIIB cells was isolated at specific time points after infection according to a modified guanidium isothiocyanate procedure. S1-mapping analysis was performed with 40 μg of RNA and 5′ [γ-^{32}P]ATP end-labeled probes specific for IFN-α1 and IFN-β as previously described (22). Nuclease-resistant DNA/RNA hybrid molecules were resolved on a 6% denaturing polyacrylamide gel, quantified by laser densitometry, and expressed as relative RNA levels.

Assay for PKR activity. PLB-985 and PLB-IIIB cells were concentrated to 10^7 cells per ml and incubated with Sendai virus (100 hemagglutinating units per ml) for 90 min at 37°C. Mock-infected cells were concentrated as described above but incubated without virus. The cells were subsequently diluted to 10^6 cells per ml and incubated for a total of 6 h. The cells were then lysed with lysis buffer (10 mM Tris-HCl [pH 7.5], 50 mM KCl, 1 mM dithiothreitol, 2 mM $MgCl_2$, 1% Triton X-100, 0.2 mM phenylmethylsulfonyl fluoride [PMSF], 3 μg of aprotinin per ml, 1 μg of leupeptin per ml, and 1 μg of pepstatin per ml). Extracts (100 μg) were incubated with anti-PKR antibody for 2 h at 4°C. Anti-mouse immunoglobulin G-agarose beads were added to the extracts and incubated for an additional 2 h at 4°C. The beads were washed four times with high-salt buffer (20 mM Tris-HCl [pH 7.5], 400 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 1% Triton X-100, 0.2 mM PMSF, 3 μg of aprotinin per ml, 1 μg of leupeptin per ml, 1 μg of pepstatin per ml, 20% glycerol) followed by three washes with low-salt buffer (10 mM Tris-HCl [pH 7.5], 100 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, 0.2 mM PMSF, 3 μg of aprotinin per ml, 1 μg of leupeptin per ml, 1 μg of pepstatin per ml, 20% glycerol). PKR activity was induced by incubating the beads with 0.1 μg of poly (rI):poly (rC) (Pharmacia) per ml in 5× kinase buffer (50 mM Tris-HCl [pH 7.7], 250 mM KCl, 10 mM $MgCl_2$, 25 mM β-mercaptoethanol) for 10 min on ice. The beads were then incubated at 30°C for 30 min with [γ-^{32}P]ATP (ICN) to detect autophosphorylation. PKR was solubilized with 2× sodium dodecyl sulfate (SDS) sample buffer and run on an 8% polyacrylamide gel. The gel was fixed with 10% methanol–5% acetic acid for 30 min, dried, and exposed to X-ray film. The intensities of the PKR bands detected by autoradiography were quantified by laser densitometry (Pharmacia).

Northern (RNA) blot analysis. Total RNA (10 to 20 μg) was electrophoresed in a 1% denaturing formaldehyde agarose gel and transferred to a nylon membrane (Hybond-N; Amersham) with a Vacu-Gene blotting system (Pharmacia). RNA was cross-linked to the membrane in a UV Stratalinker 2400 (Stratagene, La Jolla, Calif.) and prehybridized overnight in prehybridization solution {25 mM KPO_4 (pH 7.4), 5× SSC (750 mM NaCl, 75 mM sodium citrate [pH 7.0]), 50% deionized formamide, 50 μg of salmon sperm DNA (Boehringer GmbH, Mannheim, Germany)} and 5× Denhardt's solution containing 0.5% Ficoll 400 (Sigma), 0.5% (wt/vol) polyvinylpyrrolidine, and 0.5% (wt/vol) bovine serum albumin (BSA) at 42°C in a hybridization oven. The probes used included a 0.8-kb PKR fragment generated by *Eco*RI digestion of plasmid p68wt-pcDNAI/NEO (26), a 1.1-kb fragment isolated from pSVK3-IκBα by *Eco*RI digestion (6), and a 1.1-kb fragment derived from pβ-actin by *Pst*I digestion. The probes were labeled to approximately 10^9 cpm/μg with the oligolabeling kit (Pharmacia) and [α-^{32}P]CTP. Hybridization occurred overnight at 42°C in prehybridization solution supplemented with 10% (wt/vol) dextran sulfate (Pharmacia) and approximately 5×10^7 cpm of labeled probe. The blots were washed, wrapped in plastic, and exposed to X-Omat film (Kodak) at −70°C.

Western blot analysis of NF-κB/IκB proteins. Whole-cell extracts were prepared by resuspension in Nonidet P-40 (NP-40) lysis buffer (10 mM Tris-HCl [pH 8.0], 60 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, 0.5% Nonidet P-40, 0.5 mM PMSF, 0.01 mg each of leupeptin, pepstatin, and aprotinin per ml). After incubation on ice for 10 min, cellular debris was removed by centrifugation for 10 min at 4°C. Cell extracts (10 to 20 μg) were resolved by SDS-polyacrylamide gel electrophoresis (PAGE) and then transferred to a nitrocellulose membrane. The membranes were blocked for a minimum of 2 h in 5% skim milk and incubated overnight in 5% milk containing affinity-purified, anti-peptide polyclonal antisera (dilutions ranged from 1:250 to 1:1,000). Antisera were prepared as described previously (43). Antibody AR27 or anti-p105-N was raised against a peptide (amino acids 2 to 15) at the most-N-terminal region of p105 and recognizes both p105 and p50; AR43 or anti-p100-N was generated against a peptide at the N terminus of p100 (amino acids 2 to 17) and recognizes both p100 and p52; AR28 recognizes the C terminus of p65 (amino acids 537 to 550); AR22 recognizes the C terminus of c-Rel (amino acid 573 to 587). AR20 recognizes the N terminus of IκBα (aa2 to 16). Antiserum that recognizes the N terminus of RelA(p65) was purchased from Santa Cruz, Inc. The membranes were rinsed four times in phosphate-buffered saline (PBS) and incubated with a secondary antibody, fluorescein isothiocyanate-conjugated goat anti-rabbit (1:1,000; Amersham) for 1 h at room temperature and then rinsed again four times in PBS. The enhanced chemiluminescence-Western blotting (immunoblotting) detection system (Amersham) was used according to the manufacturer's instructions to visualize the specific signals. Autoradiograms were scanned by laser densitometry, and the intensity of each band is presented relative to the levels in uninfected, unstimulated cells for NF-κB subunits; quantification of IκBα was expressed as a percentage of the IκBα remaining relative to the amount at time zero. Values for turnover rates were plotted on a semi-log scale, and the best fit curves were determined. The experiments were repeated a minimum of three times, and representative autoradiograms are shown.

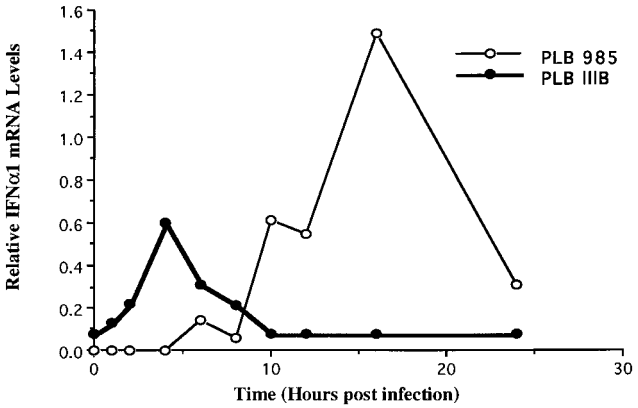

FIG. 1. Kinetics of IFN mRNA expression in PLB-985 and PLB-IIIB cells after infection with Sendai paramyxovirus. Total cellular RNA was extracted from PLB-985 and PLB-IIIB cells at hours 0, 1, 2, 4, 6, 8, 10, 12, 16, and 24 following Sendai virus infection. S1 mapping was performed with 40 μg of cellular mRNA and an [γ-^{32}P]ATP end-labeled IFN-α1- and IFN-β-specific probes, as previously described (22, 51); signals corresponding to RNA-DNA hybrids were identified by autoradiography, and the intensities of the bands corresponding to the IFN-α1 and IFN-β signals were scanned by laser densitometry and plotted in terms of relative mRNA levels. The kinetics of IFN-α1 are illustrated and are qualitatively similar to results obtained for IFN-β.

Detection of IκBα phosphorylation. Cell extracts were prepared as described for Western blot analysis and treated with 0.5 U of potato acid phosphatase in PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)] buffer (10 mM PIPES [pH 6.0], 0.5 mM PMSF, 5 μg each of leupeptin, pepstatin, and aprotinin per ml) or with 0.5 U of potato acid phosphatase in PIPES buffer plus inhibitor mix (10 mM NaF, 1.5 mM Na_2MoO_4, 0.4 mM Na_3VO_4, 0.1 μg of okadaic acid per ml) at 37°C for 60 min. Equivalent amounts of protein (50 μg) were resolved on a 5 to 13% gradient SDS-polyacrylamide gel and were transferred to nitrocellulose. IκBα and phosphorylated IκBα were visualized with the previously described affinity-purified IκBα antibody (AR20). In other experiments, PLB-985 and PLB-IIIB cells in log phase growth were concentrated to 10^6 cells per ml and were incubated with 25 μM MG132 (Myogenics) or with an equal volume of dimethyl sulfoxide (DMSO) for 0 to 120 min, as indicated. Cell extracts were electrophoresed on a SDS-5 to 13% polyacrylamide gradient gel and transferred to nitrocellulose for immunoblot analysis. Phosphorylated IκBα was detected as a distinct more slowly migrating band with a monoclonal IκBα peptide antibody (49).

RESULTS

Altered kinetics of IFN gene expression. HIV infection leads to the dysregulation of numerous cytokine genes (53). To examine whether IFN production was affected, a kinetic analysis of IFN-α1 and IFN-β gene transcription was performed with PLB-985 and PLB-IIIB cells. S1-mapping analysis with probes corresponding to IFN-β and IFN-α1 demonstrated that chronically infected PLB-IIIB cells constitutively expressed low levels of IFNα1 mRNA and IFN-β mRNA (51, 52). When coinfected with Sendai virus, PLB-IIIB cells exhibited an altered pattern of IFN gene expression compared with de novo Sendai virus infection of PLB-985 cells (Fig. 1). Sendai virus-induced IFN mRNA from PLB-IIIB cells increased at 1 h postinfection (p.i.), reached a peak at 4 to 6 h p.i., and then decreased to constitutive levels by 10 h p.i. In contrast, Sendai virus-induced IFN mRNA from PLB-985 cells was initially detected at 6 h p.i. and reached a peak at 10 to 16 h p.i. (Fig. 1). This experiment demonstrated three distinct alterations in IFN induction: (i) a constitutive level of transcription in chronically HIV-1-infected PLB-IIIB cells; (ii) rapid induction kinetics in Sendai virus-infected PLB-IIIB compared with that in PLB-985 cells; and (iii) a lower relative amount of IFN mRNA in Sendai virus-induced PLB-IIIB cells than in PLB-985 cells.

Analysis of PKR expression and activity. The dsRNA-dependent protein kinase (PKR) is induced by IFN and activated in the presence of natural or synthetic dsRNA. Since recent studies demonstrated that PKR activated NF-κB DNA-binding activity in cellular extracts (27, 30), we sought to examine whether PKR activity may be elevated in HIV-1-infected cells and may play a role in IκBα phosphorylation and turnover. Northern blot analysis demonstrated a constitutive level of PKR mRNA expression in PLB-IIIB cells (Fig. 2A, lanes 4 to 6) compared with that in PLB-985 cells (Fig. 2A, lanes 1 to 3). Also, the expression of PKR mRNA in PLB-IIIB cells increased in response to PMA for 8 h (Fig. 2A, lane 5) but not in response to Sendai virus infection (Fig. 2A, lane 6). In other experiments, PKR mRNA levels peaked at 24 h p.i. in PLB-985 cells, while in PLB-IIIB PKR mRNA levels were constitutively elevated (data not shown). Similarly, the level of PKR protein was increased by more than fivefold in PLB-IIIB cells com-

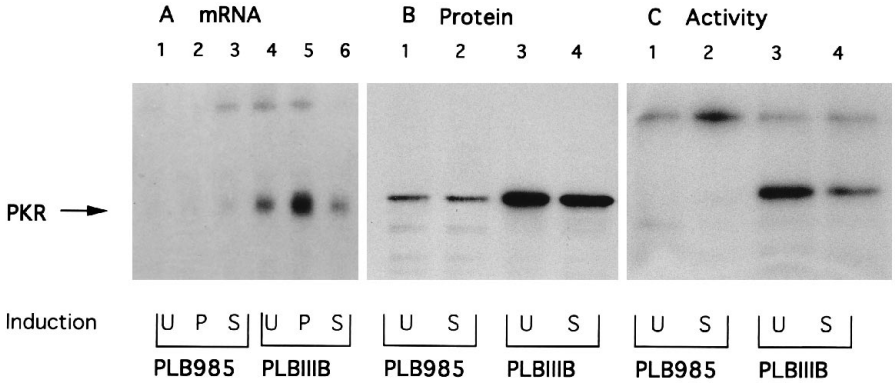

FIG. 2. Analysis of PKR mRNA and protein expression levels in PLB-985 and PLB-IIIB cells. (A) Total cellular RNA was isolated from PLB-985 (lanes 1 to 3) or PLB-IIIB (lanes 4 to 6) cells that were untreated (U), PMA treated (P), or Sendai virus infected (S) for 8 h. RNA was separated on a 1% denaturing agarose gel containing ethidium bromide and visualized under UV light (310 nm). After RNA was transferred to a nylon membrane, the blot was probed with a PKR cDNA probe. The position of the 2.5-kb PKR RNA transcript is indicated by an arrow. (B) PLB-985 cells (lanes 1 and 2) or PLB-IIIB cells (lanes 3 and 4) that were untreated (U) or infected by Sendai virus for 6 h were separated by SDS-PAGE, transferred to a nitrocellulose membrane, and immunoblotted with a monoclonal PKR antibody. The arrow indicates the band corresponding to the 68-kDa PKR protein. (C) PLB-985 cells (lanes 1 and 2) or PLB-IIIB cells (lanes 3 and 4), untreated (U) or infected by Sendai virus for 6 h (S), were immunoprecipitated with a polyclonal antisera against PKR. PKR activity was induced by poly (rI):poly(rC) and incubated with [γ-^{32}P]ATP to detect autophosphorylation. The signal was visualized by autoradiography.

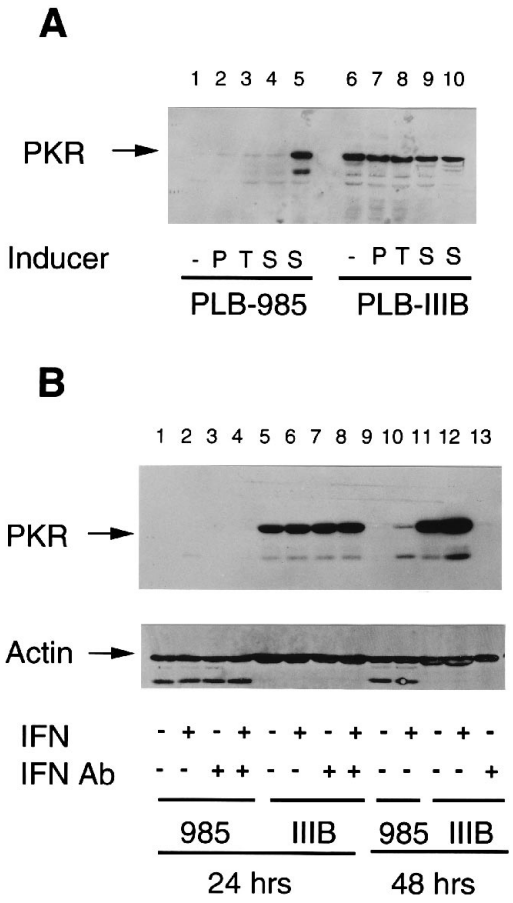

FIG. 3. Modulation of PKR protein expression levels in PLB-985 and PLB-IIIB cells. (A) PLB-985 and PLB-IIIB cells were treated with PMA (P) or TNF-α (T) or were Sendai (S) virus infected for 0 h (lanes 1 and 6), 6 h (lanes 2 to 4 and 7 to 9), or 24 h (lanes 5 and 10). PKR protein expression is identified by an arrow. (B) PLB-985 and PLB-IIIB cells were grown in the presence or absence (+ or −, respectively) of IFN or anti-IFN-α/β (IFN-Ab) for 24 or 48 h. The immunoblot was reprobed for actin to confirm equal protein loading per lane. PKR and actin are identified by arrows.

pared with that in PLB-985 (Fig. 2B). Finally, analysis of PKR autocatalytic activity demonstrated that PKR activity was increased in extracts from PLB-IIIB cells compared with that in PLB-985 cells (Fig. 2C). Darker exposures revealed that PKR activity was detectable in PLB-985 cell extracts stimulated by Sendai virus infection (data not shown). These results demonstrate that HIV-infected myeloid cells have increased PKR activity compared with that in similarly treated PLB-985 cells.

Addition of neutralizing IFN antibody downregulates PKR levels. Kinetic analysis revealed that PKR protein levels were inducible in PLB-985 but were essentially constitutive in PLB-IIIB cells (Fig. 2 and 3A). Protein levels were increased in PLB-985 cells at 18 to 24 h p.i. (Fig. 3A, lane 5) but only weakly elevated at 6 h p.i. (Fig. 3A, lanes 2 to 4). In contrast, PLB-IIIB cells had high PKR levels at various time points between 0 and 48 h after treatment with different activators (Fig. 3A, lanes 6 to 10, and Fig. 3B, lanes 5 to 9, 11 and 12). Since IFN expression may lead to elevated levels of PKR in chronically infected cells, abrogating IFN signaling by the addition of a neutralizing antibody may decrease PKR expression. To determine whether anti-IFN-α/β antibody would affect PKR expression levels in PLB-IIIB cells, 1,000 NU of anti-leukocyte IFN (anti-IFNα/β) antibody per ml was added to the cultures. PLB-IIIB cells incubated in the presence of anti-IFN antibody for 48 h had dramatically reduced levels of PKR protein (Fig. 3B, lanes 11 to 13); this effect of anti-IFN antibody required more than 24 h to be established, since changes in PKR levels were not detected in PLB-IIIB cells after 24 h of treatment with anti-IFN antibody (Fig. 3B, lanes 8 and 9). Together, these results demonstrate that chronic HIV-1 infection of PLB-985 cells resulted in low-level IFN production, which in turn stimulated constitutive expression of PKR.

IκBα turnover in PLB-985 and PLB-IIIB cells. Next, the turnover of IκBα in PLB-985 and PLB-IIIB cells was examined by immunoblot analyses (Fig. 4). Although IκBα levels were slightly elevated in HIV-1-infected cells compared with those in uninfected cells, the turnover rates were consistently higher in PLB-IIIB cells (compare PLB-985 and PLB-IIIB panels). These data were all normalized to the β-actin protein levels to ensure that overall protein degradation rates were not affected in the different extract preparations. In HIV-1-infected cells, immunoblot analysis of IκBα levels demonstrated that the total pool of IκBα turned over with a half-life of about 95 min in cycloheximide-treated cells, whereas in uninfected cells the $t_{1/2}$ of IκBα was about 165 min (compare Fig. 4A, lanes 1 to 6 for PLB-985 and PLB-IIIB; plotted in Fig. 4B). PMA-plus-cycloheximide treatment reduced the half-life of IκBα in PLB-985 and PLB-IIIB cells to 100 and 50 min, respectively (Fig. 4A, lanes 7 to 12; Fig. 4B). Treatment of both PLB-985 and PLB-IIIB with TNF-α in the presence of cycloheximide resulted in rapid inducer-mediated degradation of IκBα with a $t_{1/2}$ of <10 min (Fig. 4, lanes 13 to 18; Fig. 4B). Interestingly, a 30-kDa AR20 immunoreactive band exhibiting degradation kinetics similar to that of IκBα was present in PLB-985 cells but not in PLB-IIIB cells. The 30-kDa form appears to be a degradation product of IκBα, although we cannot rule out the possibility of a differentially spliced product or a distinct immunoreactive species. Similar turnover kinetics for IκBα in U937 cells and HIV-1-infected U9-IIIB cells were also observed (Fig. 5). In the presence of cycloheximide, IκBα had half-lives of approximately 120 and 80 minutes in U937 cells and U9-IIIB cells, respectively (compare Fig. 5A, lanes 1 to 6 for U937 and U9-IIIB; Fig. 5B). As seen with PLB-985 and PLB-IIIB cells, TNF-α reduced the turnover rate to <10 min for both cell types (Fig. 5A, lanes 7 to 12; Fig. 5B).

Interestingly, the addition of anti-IFN antibody to the culture medium for 48 h altered the kinetics of IκBα turnover in PLB-IIIB cells (Fig. 6). Immunoblot analysis of IκBα levels demonstrated that the total pool of IκBα degraded with a half-life of about 90 min in cycloheximide-treated PLB-IIIB cells, whereas in HIV-1-infected cells treated with anti-IFN antibody, the $t_{1/2}$ of IκBα was about 160 min, similar to the turnover rate in uninfected PLB-985 cells. These results indicate that constitutive PKR activity in HIV-1-infected cells may contribute to the overall turnover of the IκBα pool and that downregulation of PKR by interfering with the IFN-inductive signal may alter IκBα turnover in PLB-IIIB cells.

IκBα mRNA expression in PLB-985 and PLB-IIIB cells. We next sought to determine if the increased turnover rate of IκBα in HIV-infected cells was accompanied by increased transcription of the IκB gene, as described previously (28). IκBα mRNA expression was examined by Northern blot analyses and normalized to actin levels (Fig. 7). IκBα (MAD-3) mRNA levels were equivalent in unstimulated PLB-985 and PLB-IIIB cells normalized to actin (Fig. 7A, lanes 1 and 2 and 6 and 7) and were inducible by PMA treatment or Sendai virus infection by approximately 25-fold in PLB-985 and by 5- to 15-fold in PLB-IIIB (Fig. 7, lanes 3 to 5 and 8 to 10). In other exper-

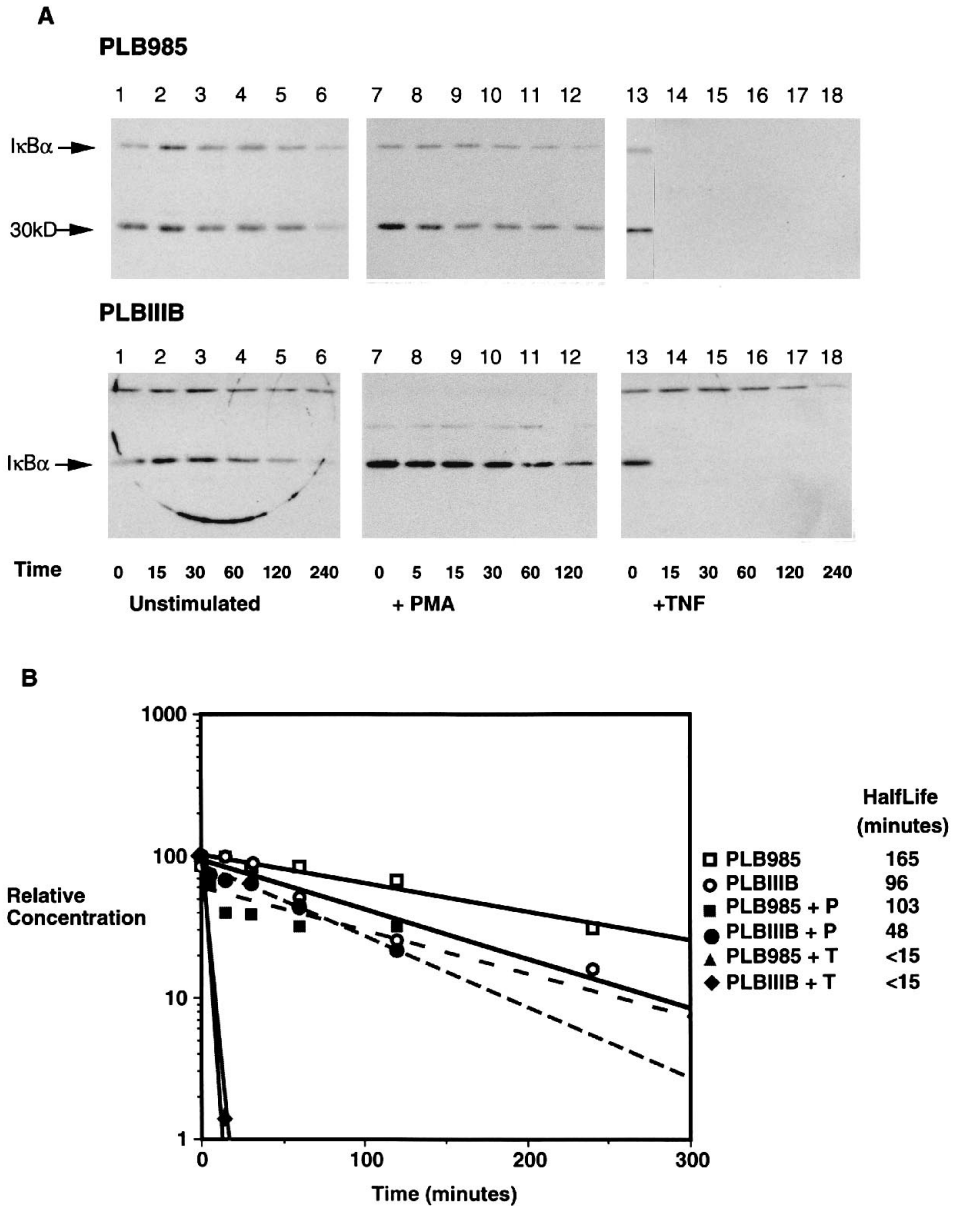

FIG. 4. IκBα turnover in PLB-985 and PLB-IIIB cells. (A) PLB-985 and PLB-IIIB cells were cultured in the presence of cycloheximide and were either untreated, PMA treated, or TNF-α treated for the times (in minutes) indicated. Protein extracts were resolved by SDS-PAGE, transferred to nitrocellulose, and visualized with affinity-purified IκBα antibody (AR20). The band corresponding to IκBα is identified by an arrow. (B) Autoradiograms were scanned by laser densitometry and the intensity of each band is presented in terms of the percentage of IκBα remaining in cells untreated or treated with either PMA (P) or TNF-α (T) compared with the total IκBα at time zero.

iments, IκBα mRNA levels were maximally stimulated 2 h after treatment in PLB-IIIB cells, and by 24 h after induction, IκBα mRNA levels were the same as those in unstimulated cells (data not shown). Thus, in response to increased NF-κB binding activity in HIV-1-infected cells, the IκBα/MAD-3 gene was transcriptionally induced in both cell types, although with different kinetic profiles. Also, in PLB-IIIB cells, induction of IκBα transcription appears to be decreased in response to various stimuli (Fig. 7). This modulation in the level of IκBα gene induction may reflect differences in the relative abundance and transactivation potential of the constitutive NF-κB binding activity in HIV-1 infected cells (see below).

Phosphorylation of IκBα. To examine the comparative degree of phosphorylation of IκBα in PLB-985 and PLB-IIIB, IκBα was analyzed at different times following treatment with TNF-α or PMA (Fig. 8). Phosphorylated IκBα was detected by immunoblot analysis in both PLB-985 cells (data not shown) and PLB-IIIB cells treated with TNF-α as early as 1 or 2 min after stimulation (Fig. 8A, lanes 2 and 3). The more slowly migrating band, present only in TNF-α-stimulated cells, was lost when extracts were incubated with potato acid phosphatase (Fig. 8A, lane 5) and was restored when extracts were incubated with phosphatase and phosphatase inhibitors (Fig. 8A, lane 6), thus confirming that the upper band represents a phosphorylated form of IκBα. PMA treatment for 4 min did not result in the appearance of phosphorylated IκBα (Fig. 8A, compare lanes 4 and 2), indicating that TNF-α is a more potent inducer of IκBα phosphorylation than is PMA. This observa-

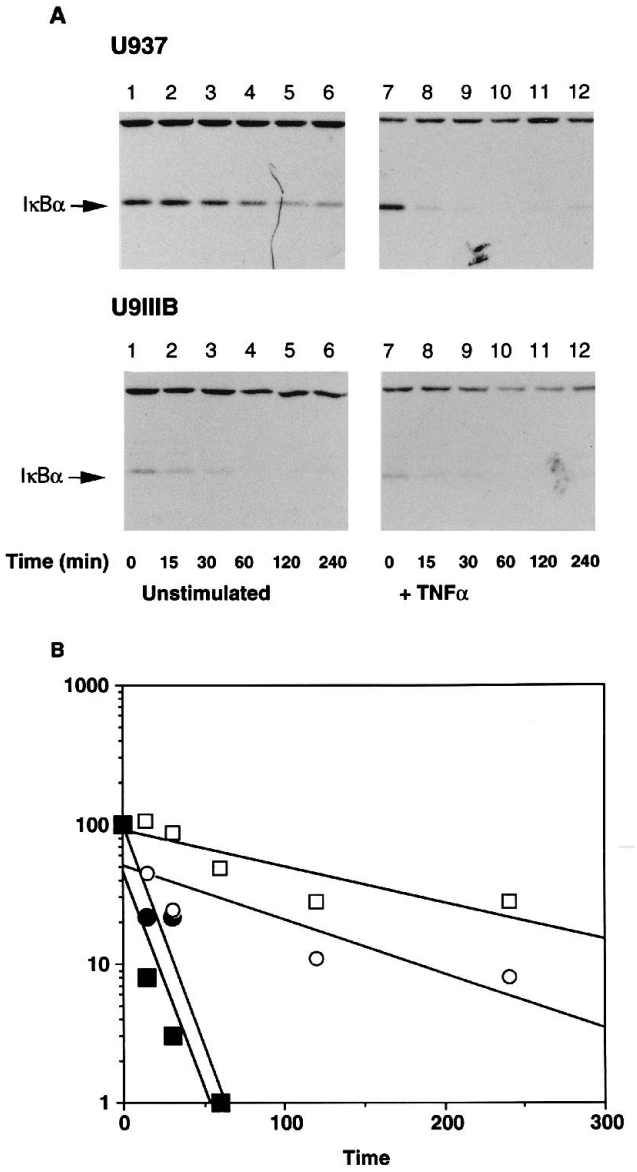

FIG. 5. IκBα turnover in U937 and U9-IIIB cells. (A) U937 and U9-IIIB cells were cultured in the presence of cycloheximide and were either untreated or TNF-α treated for the times (in minutes) indicated. Protein extracts were resolved by SDS-PAGE, transferred to nitrocellulose, and visualized with affinity-purified IκBα antibody (AR20). The band corresponding to IκBα is identified by an arrow. (B) Autoradiograms were scanned by laser densitometry, and the intensity of each band is presented in terms of the percentage of IκBα remaining in U937 ($t_{1/2}$, 120 min) (□), U937 plus TNF-α ($t_{1/2}$, <10 min) (■), U9-IIIB ($t_{1/2}$, 80 min) (○), and U9-IIIB plus TNF-α ($t_{1/2}$, <10 min) (▨) cells compared with the total percentage at time zero.

tion is also consistent with the limited effect of PMA on IκBα turnover (see Fig. 4).

Constitutive turnover of IκBα protein was increased in PLB-IIIB compared with that in PLB-985 cells (see Fig. 4). To determine if this increase was related to an enhanced constitutive phosphorylation in HIV-1-infected cells, the rate of accumulation of phosphorylated IκBα was analyzed in the presence of the proteasome inhibitor MG132 (Fig. 8B). By this approach, IκBα phosphorylation in PLB-985 (Fig. 8B, lanes 1 to 6) and PLB-IIIB (Fig. 8B, lanes 8 to 13) appeared to be similar. Also, extracts from PLB-985 or PLB-IIIB cells (Fig. 8B, lanes 7 or 14) incubated with DMSO for 2 h resembled control extracts, confirming that accumulation of phosphorylated IκBα was not due to DMSO treatment but rather to stabilization of the phosphorylated product by MG132. Interestingly, the 30-kDa IκBα-like band specific to PLB-985 was phosphorylated at time zero (Fig. 8B, lane 1, lower band); at later times, two distinct slowly migrating forms of 30-kDa IκBα were detectable (Fig. 8B, lanes 5 and 6).

NF-κB/Rel protein expression in PLB-985 and PLB-IIIB cells. The altered kinetics of IκBα turnover in PLB-IIIB cells suggested that expression of NF-κB subunits may be similarly altered in HIV-1-infected cells. In previous studies the composition of NF-κB-DNA complexes was analyzed (50, 52), but the relative abundance of different NF-κB subunits was not evaluated. To examine the protein levels of various NF-κB DNA-binding proteins in HIV-1-infected and control PLB-985 cells, immunoblot analyses with NF-κB subunit-specific antibodies were performed and normalized by analysis of β-actin levels (Fig. 9 and 10). Protein extracts were prepared from PLB-985 and PLB-IIIB cells induced for 8, 16, or 22 h with PMA or TNF-α. A representative summary of the immunoblot analyses of NF-κB1 and NF-κB2 proteins is shown in Fig. 9 and illustrates that the levels of NF-κB2 p100 and p52 (Fig. 9A and B) were higher in unstimulated PLB-IIIB cells than in unstimulated PLB-985 cells. HIV-1-infected cells had approximately three- to fourfold more p100 and twofold more p52 than uninfected PLB-985 cells. NF-κB1 p105 and p50 levels (Fig. 9C and D) were not significantly increased. Stimulation with PMA or TNF-α (Fig. 9 and data not shown) had little effect on NF-κB1 p105 and p50 levels but resulted in enhanced levels of NF-κB2 p100 (Fig. 9A). RelA(p65) levels were about

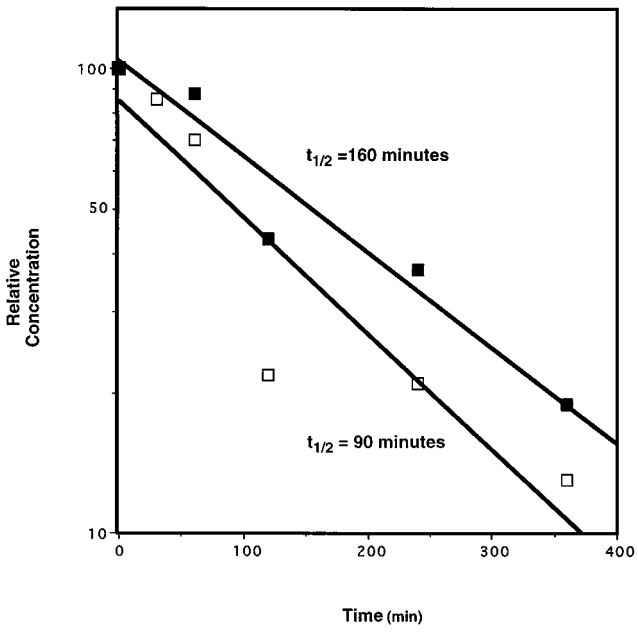

FIG. 6. IκBα turnover in PLB-IIIB cells treated with anti-IFN antibody. PLB-IIIB cells were either untreated or treated with anti-IFN antibody at 1,000 IU/ml for 48 h. Cells were then cultured in the presence of cycloheximide for different times, protein extracts (20 μg) were resolved by SDS-PAGE, transferred to nitrocellulose, and visualized with affinity-purified IκBα antibody (AR20), as described in the legend to Fig. 4. Autoradiograms were scanned by laser densitometry, and the intensity of IκBα remaining at different times after the cycloheximide addition is presented as percentage of IκBα remaining in untreated PLB-IIIB cells (□) or PLB-IIIB cells treated with anti-IFN antibody (▨).

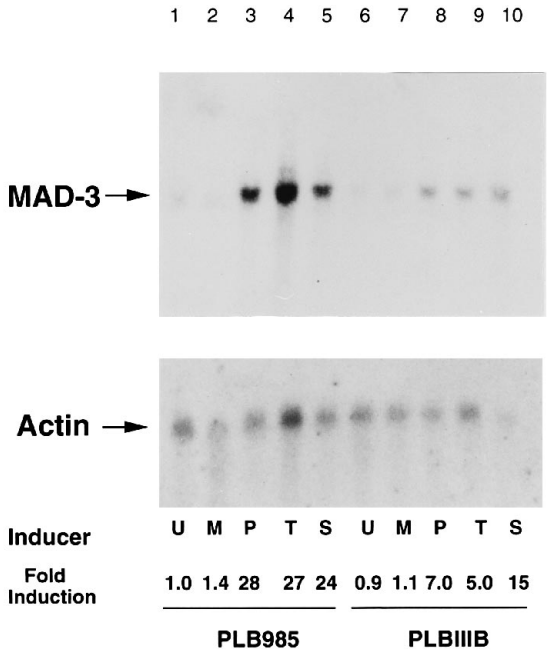

FIG. 7. Northern blot analysis of IκBα (MAD-3) mRNA levels in PLB-985 and PLB-IIIB cells. Total cellular RNA was isolated from PLB-985 (lanes 1 to 5) and PLB-IIIB (lanes 6 to 10) cells that were untreated (U), mock infected (M), PMA treated (P), TNF-α (T) treated, or Sendai virus infected (S) for 6 h. RNA was separated on a 1% denaturing agarose gel containing ethidium bromide and visualized under UV light (310 nm). After RNA was transferred to a nylon membrane, the blot was first probed with an IκBα cDNA probe. The blot was subsequently stripped and reprobed with an actin cDNA probe. The positions of the IκBα RNA transcript MAD-3 (top panel) and the actin transcript (lower panel) are identified by arrows. The fold induction after normalization to the actin signal is indicated below each lane.

twofold higher in PLB-IIIB cells than in PLB-985 cells, and the levels were not dramatically altered by PMA or TNF-α treatment (Fig. 10C and D). In contrast, the levels of c-Rel were at least eightfold higher in infected cells than in noninfected cells (Fig. 10A and B). Following induction with PMA or TNF-α, c-Rel levels were up to 50-fold higher than the levels detected in unstimulated PLB-985 cells and 2- to 3-fold higher than the levels of c-Rel protein in unstimulated PLB-IIIB cells (Fig. 10A and B). Interestingly, stimulation with PMA or TNF-α for 22 h (Fig. 10B and data not shown) led to sustained expression of c-Rel in PLB-IIIB cells, whereas in PLB-985 cells, c-Rel levels returned to control levels. Other studies also demonstrated that NF-κB subunit expression was similarly increased in U9-IIIB cells and in U937 cells; in addition, U9-IIIB cells exhibited increased levels of p105 and p50 (data not shown). These results suggest that intracellular pools of NF-κB subunits are increased in chronically HIV-1-infected myeloid cells.

DISCUSSION

In this study we demonstrate that regulation of NF-κB/IκBα expression is altered in PLB-IIIB cells as a result of chronic HIV-1 infection: (i) constitutive expression of IFN mRNA and altered kinetics of IFN mRNA induction in response to Sendai virus infection were demonstrated in PLB-IIIB cells compared with those in PLB-985 cells; (ii) PKR protein and enzymatic activity were increased in PLB-IIIB cells; (iii) IκBα turnover was increased in HIV-1-infected cells compared with that in uninfected PLB-985 cells, in part because of PKR activity; and (iv) increased levels of c-Rel and p100/p52 proteins were detected in PLB-IIIB cells compared with those in PLB-985 cells. The addition of anti-IFN-α/β antibody to the culture dramatically reduced the level of PKR expression in PLB-IIIB cells, thus demonstrating a role for low-level IFN production in the constitutive activation of PKR. As demonstrated previously, PKR is capable of phosphorylating IκBα in vitro and inducing NF-κB-binding activity (27, 30). The persistent inductive signal provided by PKR may contribute to the constitutive NF-κB-binding activity observed in HIV-1-infected myeloid cells. On the basis of the selective increase in NF-κB subunits, nuclear translocation of NF-κB appears to ultimately increase the intracellular pool of NF-κB/IκBα by an autoregulatory mechanism. The increased pool of NF-κB/IκBα may contribute to the chronically activated state of HIV-1-infected cells.

Several studies have addressed the mechanism of NF-κB activation in HIV-1-infected cells. Bachelerie et al. demonstrated sustained NF-κB binding activity in the chronically HIV-infected promonocytic cell line U937 (2); furthermore, occupancy of the viral enhancer by NF-κB (p50/p65) was re-

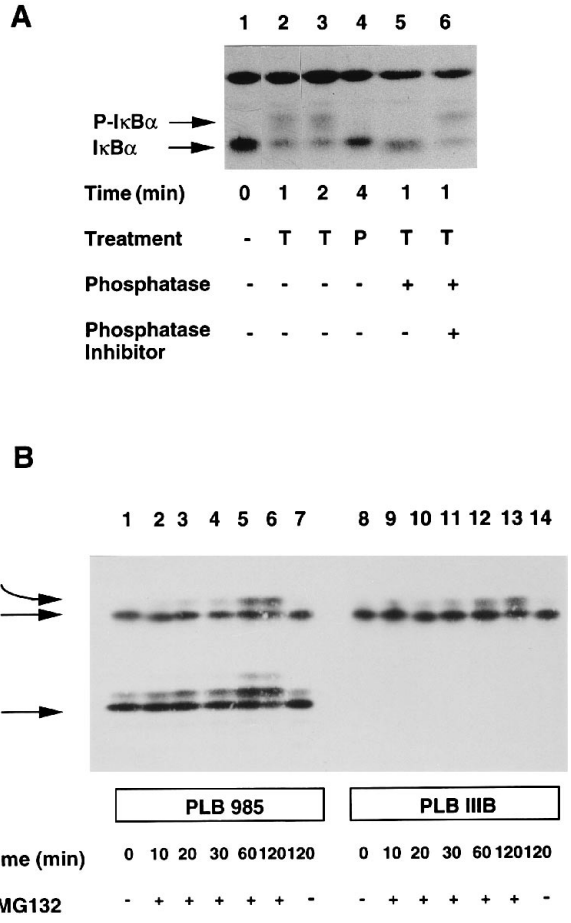

FIG. 8. Analysis of IκBα phosphorylation. (A) PLB-IIIB cells were stimulated with TNF-α (T) for 1 or 2 min (lanes 2 or 3, respectively) or PMA (P) for 4 min (lane 4). Cell extracts were prepared, and TNF-α-treated samples (1 min) were then subjected to potato acid phosphatase treatment (lane 5) or potato acid phosphatase treatment plus inhibitors (lane 6) for 30 min. Equal amounts of protein were separated by SDS-PAGE, transferred to nitrocellulose, and visualized with affinity-purified IκBα antibody (AR20). TNF-α-induced phosphorylation was compared with control levels (lane 1). (B) The accumulation of phosphorylated IκBα (P-IκBα) was analyzed in PLB-985 cells (lanes 1 to 7) and PLB-IIIB cells (lanes 8 to 14) incubated with 25 μM MG132 (lanes 2 to 6 and 9 to 13) or DMSO (lanes 7 and 14) for the indicated times. IκBα, P-IκBα, and the 30-kDa band, detected with a monoclonal IκBα antibody, are indicated by arrows.

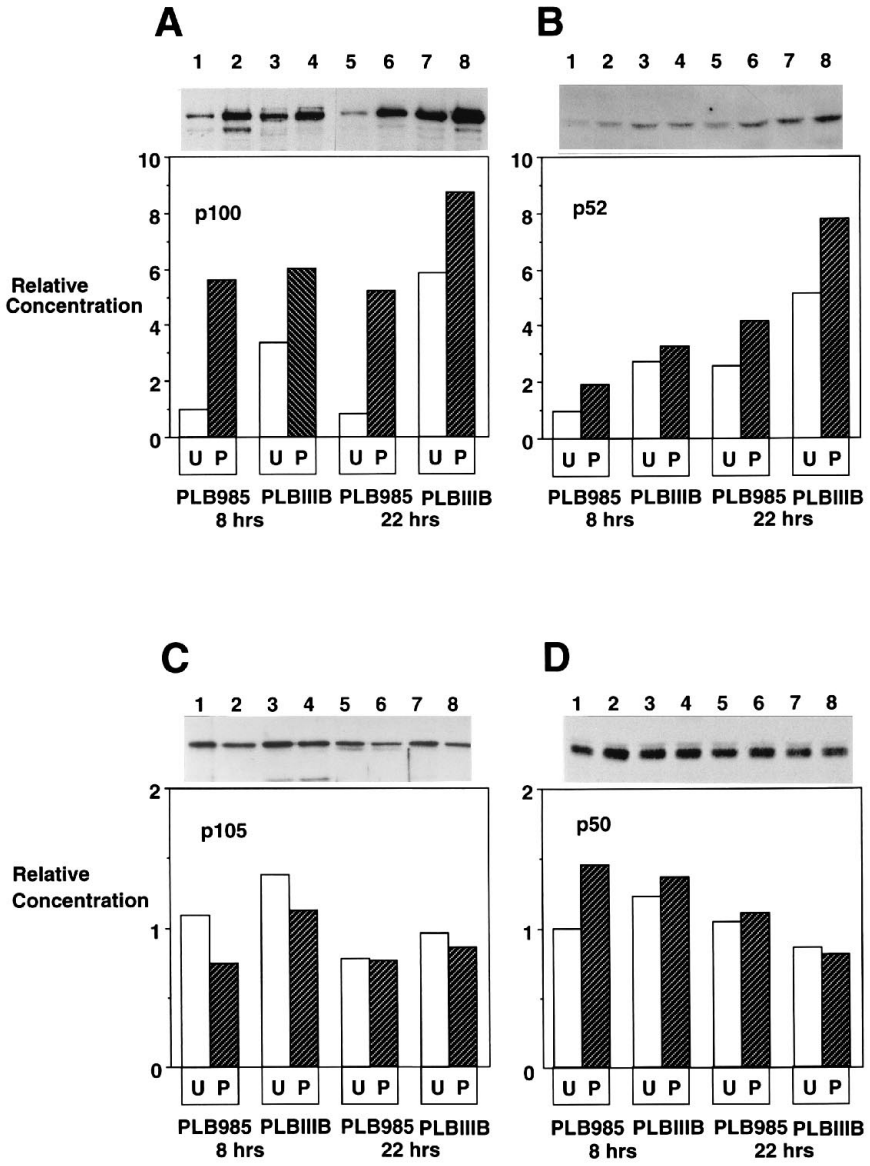

FIG. 9. Analysis of NF-κB1 and NF-κB2 protein expression in PLB-985 and PLB-IIIB cells. Cell extracts isolated by detergent lysis from PLB-985 and PLB-IIIB cells left untreated (□) or stimulated with PMA for 8 or 22 h (▨) were resolved by SDS-PAGE, transferred to a nitrocellulose membrane, and blotted with NF-κB-specific antisera: p100 (AR40) (A), p52 (AR43) (B), p105 (AR27) (C), and p50 (AR27) (D). The specific signals were detected with the enhanced chemiluminescence detection system (Amersham). Autoradiograms were scanned by laser densitometry, and the intensity of the each band is plotted in terms of relative protein levels. Because of affinity differences between NF-κB antibodies, the relative amounts of the different proteins cannot be compared.

quired for ongoing transcription of integrated HIV provirus in monocytic cells chronically infected with HIV-1 (24a). Increased transcription and processing of the p105 precursor may be one mechanism to increase intracellular pools of NF-κB in chronically infected U937 cells (42) and may be mediated by the HIV protease which cleaves p105 to generate a 45-kDa DNA-binding subunit in vitro and in HIV-infected T cells (48). Although we also found elevated expression of p105 protein in chronically infected U937 cells, significant increases in p105 protein were not seen in PLB-IIIB cells. These cells, although both myeloid precursor cells, differ in their state of differentiation, suggesting that NF-κB may be developmentally regulated.

HIV infection of the monocytic THP-1 cell line resulted in two populations of chronically infected cells (47). Nuclear extracts from productively infected cells contained increased p50-p65 heterodimer-binding activity, whereas extracts from cells expressing low levels of virus (restricted expression) displayed only p50 homodimer-binding activity. In another study, increased NF-κB p50-p65-binding activity was also observed in HIV-1-infected peripheral blood mononuclear cells and macrophages but not in the promonocytic cell line U937, suggesting that a certain level of myeloid maturation is required prior to NF-κB induction (60).

The activity of specific lysosomal proteases expressed in myelomonocytic cells, including primary monocytes, HL-60, and certain clones of U937 and THP-1 cells, resulted in carboxy-terminal cleavage of RelA(p65). The generation of a truncated form of RelA resulted in the formation of Rel-A containing protein-DNA complexes with transcriptional activity that re-

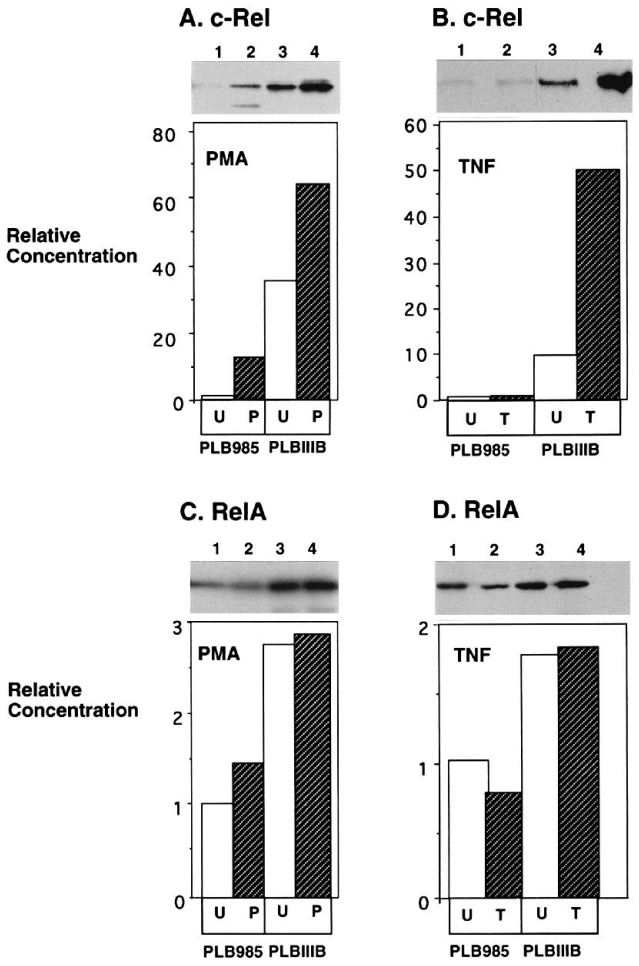

FIG. 10. Analysis of c-Rel and RelA(p65) expression in PLB-985 and PLB-IIIB cells. Cell extracts isolated by detergent lysis from PLB-985 and PLB-IIIB cells left untreated (U) (□), treated with PMA (P) for 16 h, or treated with TNF-α (T) for 22 h (▨) were resolved by SDS-PAGE, transferred to a nitrocellulose membrane, and blotted with NF-κB-specific antisera: c-Rel with and without PMA (AR22) (A), c-Rel with and without TNF-α (B), p65 with and without PMA (AR28) (C), and p65 with and without TNF-α (AR28) (D).

sembled NF-κB1 p50 homodimers (18). The activity of lysosomal proteases was decreased after cellular exposure to agents that induced monocytic but not granulocytic differentiation. Although RelA cleavage was thought to occur only during cell extract preparation in vitro, this enzymatic activity may nonetheless restrict virus expression in immature myeloid cells in vivo, by a mechanism independent of transcription, thus accounting for cell populations exhibiting restricted or productive patterns of HIV-1 replication (18). An N-terminal RelA-specific antibody was used to examine whether PLB-IIIB or PLB-985 cells contained the truncated form of RelA; no evidence of truncated RelA was obtained with the PLB-IIIB/PLB-985 extracts or isolation procedure used in this study (data not shown).

Modulation of intracellular NF-κB protein levels represents a control mechanism for enhanced activation of NF-κB-regulated gene expression. Enhanced levels of NF-κB2 and c-Rel in PLB-IIIB (and NF-κB2, NF-κB1, and c-Rel in U9-IIIB) cells may result in increased intracellular pools of latent NF-κB. c-Rel expression was particularly augmented in HIV-infected cells and exhibited increased responsiveness to PMA and TNF-α stimulation. We previously reported the appearance of an unidentified 90- to 100-kDa protein upon HIV infection of PLB-985 cells. This protein bound the IFN-β PRDII NF-κB site with high affinity (50–52) as well as the HIV-1 NF-κB sequence. Given the dramatic increase in c-Rel activity and the induction of c-Rel in response to TNF-α and PMA stimulation, these results raise the possibility that the 90-kDa protein is in fact c-Rel (50–52). The *relA*(p65) gene is not known to be stimulated in response to mitogenic stimuli, including PMA (56), and is not regulated by NF-κB (62). Our results are consistent with previous observations, since the levels of p65 were only moderately increased in the differentiated PLB-IIIB cells. Increasing the intracellular levels of distinct NF-κB subunits would permit high levels of HIV long terminal repeat-directed gene transcription and would thus provide an environment for continued HIV replication (24a). Furthermore, changing the relative abundance of NF-κB would also be expected to alter host transcription since several studies have delineated differential transcriptional specificities of NF-κB homo- and heterodimer combinations (reviewed in reference 53).

IκBα phosphorylation and turnover. The level of IκBα protein was consistently lower in PLB-985 cells than in PLB-IIIB cells. However, the pool of IκBα in PLB-IIIB cells exhibited a higher rate of protein turnover. Increased IκBα protein turnover has been documented in other HIV-1-infected myeloid cells (2, 31), suggesting one mechanism for increased constitutive NF-κB DNA-binding activity in HIV-1-infected cells (50). Using the proteasome inhibitor MG132, we demonstrated the accumulation of phosphorylated forms of IκBα in both uninfected and HIV-1-infected cells, although only minor differences in the rates of accumulation were detected. McElhinny et al. also suggested that the increased turnover compensated for the elevated levels of IκBα protein seen in chronically HIV-1-infected U937 cells (31). Increased IκBα synthesis and turnover, coupled with the elevated levels of NFκB2 p100/p52 and c-Rel, indicate a specific upregulation of the NF-κB/IκBα autoregulatory loop in HIV-1-infected myeloid cells. The observation that IκBα mRNA induction is decreased in PLB-IIIB cells may be explained by the differential responsiveness of the IκBα promoter to NF-κB activation. The IκBα promoter is transcriptionally activated by the NF-κB subunits RelA and p50 but to only a limited extent by c-Rel (28). While the levels of RelA are not significantly elevated in HIV-1-infected cells, c-Rel and p100/p52 account for a much greater proportion of the NF-κB pool.

Interestingly, the 30-kDa IκB-like band specific to PLB-985 cells degraded with kinetics similar to those of IκBα and exhibited a similar pattern of accumulation of phosphorylated forms in the presence of MG132. In fact, the 30-kDa protein accumulated two distinct phosphorylated species with time after the addition of the proteasome inhibitor. The characteristics of this species are consistent with a degradation product of IκBα, although it could also represent an alternatively spliced form of IκBα or a distinct cross-reactive form. Likewise, it is not known if the 30-kDa protein maintains the capacity for cytoplasmic retention of NF-κB. If the 30-kDa form is a degradation product of IκBα, then the total pool of IκBα in PLB-985 cells would be greater than the pool in PLB-IIIB cells.

Role of PKR in virus-induced NF-κB-binding activity. Both acute Sendai virus infection and chronic HIV infection lead to the induction of distinct NF-κB DNA-binding activities (50–52), suggesting that the two viruses may converge on a signaling pathway distinct from that stimulated by PMA or TNF-α in the PLB-985 cell model. One common point at which the infection cycle of these viruses may overlap is with the gener-

ation of dsRNA during viral replication, which may in turn activate PKR (34, 58). PKR is induced by IFN and is activated by binding to dsRNA molecules generated during viral infection. The antiviral activities of IFN are in part brought about by a PKR-mediated phosphorylation of initiation factor eIF-2α, which in turn inhibits protein translation. Recent work has implicated PKR in the activation of NF-κB DNA-binding activity in vitro and in vivo (27, 30). PKR phosphorylated IκBα and activated NF-κB DNA-binding activity in cellular extracts (27). In PKR-depleted HeLa cells, NF-κB DNA-binding activity was not activated by dsRNA [poly(I):poly(C)] (30). Increased PKR activity, acting upstream of IκBα phosphorylation, is a possible mechanism by which constitutive activation of NF-κB may be perpetuated in chronically infected cells. A recent study demonstrated that expression of wild-type PKR but not dsRNA-binding domain mutants of PKR stimulated HIV-1 long terminal repeat expression (35). Furthermore, PKR may induce NF-κB by two potential mechanisms: by phosphorylation of IκB or by inhibition of de novo IκB protein synthesis, thus preventing the shut-down of NF-κB-binding activity. It is likely that other kinases and signaling pathways that ultimately influence NF-κB activation are also constitutively activated in chronically HIV-1-infected cells (53).

HIV-1 Tat protein was previously shown to downregulate PKR activity in an HIV-infected T-cell line and in Tat-expressing HeLa cells (55), although this observation is not consistently observed. Direct physical association between Tat and PKR in vitro and in vivo in HeLa cells and T lymphocytes has been recently reported, suggesting a potential mechanism by which HIV could suppress PKR activity specifically and the interferon system in general (32). In contrast, our results indicate that PKR mRNA, protein, and enzymatic levels are upregulated in HIV-infected monocytic cells; low-level IFN production in HIV-1-infected cells appears to be sufficient to activate PKR activity. PKR may also be more active in HIV-infected cells since HIV TAR RNA can activate PKR kinase activity in vitro (16, 54). Further studies are underway to directly implicate PKR in the modulation of HIV-1 infection and NF-κB activation.

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