Human T-Cell Lymphotropic Virus Type 1 p12$^{\text{I}}$ Enhances Interleukin-2 Production during T-Cell Activation

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Human T-cell lymphotropic virus type 1 (HTLV-1) causes adult T-cell leukemia/lymphoma (ATLL) and a variety of lymphoproliferative disorders. The early virus-cell interactions that determine a productive infection remain unclear. However, it is well recognized that T-cell activation is required for effective retroviral integration into the host cell genome and subsequent viral replication. The HTLV-1 pX open reading frame I encoding protein, p12$^{\text{I}}$, is critical for the virus to establish persistent infection in vivo and for infection in quiescent primary lymphocytes in vitro. p12$^{\text{I}}$ localizes in the endoplasmic reticulum (ER) and cis-Golgi apparatus, increases intracellular calcium and activates nuclear factor of activated T cells (NFAT)-mediated transcription. To clarify the function of p12$^{\text{I}}$, we tested the production of IL-2 from Jurkat T cells and peripheral blood mononuclear cells (PBMC) expressing p12$^{\text{I}}$. Lentiviral vector expressed p12$^{\text{I}}$ in Jurkat T cells enhanced interleukin-2 (IL-2) production in a calcium pathway-dependent manner during T-cell receptor (TCR) stimulation. Expression of p12$^{\text{I}}$ also induced higher NFAT-mediated reporter gene activities during TCR stimulation in Jurkat T cells. In contrast, p12 expression in PBMC elicited increased IL-2 production in the presence of phorbal ester stimulation, but not during TCR stimulation. Finally, the requirement of ER localization for p12$^{\text{I}}$-mediated NFAT activation was demonstrated and two positive regions and two negative regions in p12$^{\text{I}}$ were identified for the activation of this transcription factor by using p12$^{\text{I}}$ truncation mutants. These results are the first to indicate that HTLV-1, an etiologic agent associated with lymphoproliferative diseases, uses a conserved accessory protein to induce T-cell activation, an antecedent to efficient viral infection.

Human T-cell lymphotropic virus type-1 (HTLV-1) infects approximately 15 to 25 million people worldwide (19). The viral infection causes adult T-cell leukemia/lymphoma (ATLL), an aggressive lymphoproliferative disease, and is associated with HTLV-1-associated myelopathy/tropical spastic paraparesis, as well as a variety of lymphocyte-mediated disorders (3, 14, 21, 44, 49). Although a minority (1 to 5%) of infected subjects develop these diseases, the virus persists in all infected individuals (37). HTLV-1 immortalizes and eventually transforms human primary peripheral blood T cells in vitro after long-term culture. This transformation process has been extensively investigated but remains to be incompletely understood. Activated T cells, however, are more susceptible to HTLV-1 infection compared to quiescent T cells (32). Therefore, T-cell activation appears necessary for the virus to efficiently establish infection.

The underlying mechanisms of early HTLV-1 infection and details of virus-mediated T-cell activation in vivo remain elusive. Recent studies indicate an important role of the HTLV-1 accessory protein p12$^{\text{I}}$ in viral infection and T-cell activation. p12$^{\text{I}}$ is produced throughout the viral infection, as indicated by

the production of mRNA of open reading frame (ORF) I in HTLV-1-infected cells derived from ATLL patients and asymptomatic carriers (4–7, 17, 25). Cytotoxic T lymphocytes isolated from HTLV-1-infected patients with HTLV-1-associated myelopathy/tropical spastic paraparesis or ATLL, as well as HLA-A2 asymptomatic carriers, recognize peptides representing regions of p12$^{\text{I}}$ (42). Moreover, antibodies from naturally infected subjects and experimentally infected rabbits recognize p12$^{\text{I}}$ (10). Although initial studies reported that HTLV-1 ORF I was dispensable for viral infection in vitro (11, 45), selective ablation of ORF I from a HTLV-1 proviral clone dramatically reduced viral infectivity in vivo (9). In support of this finding, ORF I deletion also reduced the viral infectivity in quiescent peripheral blood mononuclear cells (PBMC) in the absence of interleukin-2 (IL-2) and mitogen stimulation (1). The ability of the mutated virus to infect PBMC was restored when mitogen was added to the culture system. These findings indicate a critical role of p12$^{\text{I}}$ for viral infection in quiescent T lymphocytes, suggesting the potential function of the viral protein in T-cell activation.

Sequence analysis of the HTLV-1 p12$^{\text{I}}$ protein reveals two putative transmembrane regions and four putative proline-rich SH3-binding domains (14), implying the possible interactions with cell signaling pathways. p12$^{\text{I}}$ has been demonstrated to associate with the p16 subunit of the vacuolar hydrogen ATPase and has weak oncogenic properties (15). In addition, this viral protein binds the IL-2 receptor (IL-2R) β and γ chains and enhances Stat5 DNA-binding activity (35, 40). This

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interaction may be responsible for the reduced IL-2 requirement for PBMC expressing p12^I to proliferate. However, the mechanism of Stat5 activation mediated by p12^I is yet to be determined. p12^I localizes in the endoplasmic reticulum (ER) and cis-Golgi compartment and associates with an ER luminal protein, calreticulin, which regulates calcium homeostasis in a variety of cell types (13, 24). Furthermore, expression of p12^I in Jurkat T cells elevates basal intracellular calcium (12) and selectively activates in a calcium dependent manner nuclear factor of activated T cells (NFAT), a downstream transcription factor in T lymphocytes (2). As a T-cell growth factor, IL-2 is upregulated, in part, by NFAT activation and promotes T-cell proliferation.

In the present study, we report that p12^I expression via a lentiviral system enhances IL-2 production in T lymphocytes in a calcium pathway-dependent manner. Elevated NFAT transcriptional activities in T lymphocytes expressing p12^I suggest that IL-2 production is a result of increased NFAT activation. Moreover, two positive and two negative regions were identified in p12^I that regulate the NFAT activation by using truncation mutants of p12^I. Our data indicate the critical function of this viral accessory protein in T-cell activation and early events of the viral infection.

MATERIALS AND METHODS

Cells. The 293T cell line is the 293 cell line (catalog number 1573; American Type Culture Collection) which stably expresses the simian virus 40 T antigen. HeLa-Tat is a human cervical carcinoma cell line, HeLa, which stably expresses human immunodeficiency virus type 1 (HIV-1) Tat protein (obtained through the AIDS Research and Reference Reagent Program). Both 293T and HeLa-Tat were maintained in Dulbecco modified Eagle medium (DMEM; Gibco, Rockville, Md.) supplemented with 10% fetal bovine serum, 100 μg of streptomycin-penicillin/ml plus 2 mM L-glutamine (complete DMEM). Human PBMC were obtained by leukophoresis as previously described (38) and were maintained in RPMI 1640 media (Gibco) supplemented with 15% fetal bovine serum, 100 μg of streptomycin-penicillin/ml, and 2 mM L-glutamine (complete RPMI [cRPMI]). Jurkat T cells (clone E6-1; American Type Culture Collection catalog number TIB-152) were maintained in cRPMI plus 10 mM HEPES (Gibco).

Plasmids. The pME-18S and pME-p12 plasmids (35) were provided by G. Franchini (National Cancer Institute, National Institutes of Health). The pME-p12 plasmid expresses the fusion protein of HTLV-1 p12^I tagged with the influenza hemagglutinin (HA1) tag. p12^I truncation mutants generated in the pME-18S vector were previously described (13). Mutant p12^{I}15-47KKLL was constructed by inserting the KKLL sequence (20, 43), an ER localization signal, into the *Not*I and *Xba*I sites in the mutant plasmid p12^{I}15-47. p12^I point mutants (A$_8$XXA$_{11}$, A$_{35}$XXA$_{38}$, A$_{70}$XXA$_{73}$, and A$_{90}$XXA$_{93}$) were generated by changing proline sequence to alanine sequence in plasmid pME-p12 by using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, Calif.). The plasmids pHR′CMV/p12(+)/eGFP and pHR′CMV/p12(−)/eGFP were generated by replacing the tax sequence in plasmid pHR′CMV/Tax1/eGFP (53) with the sense p12^I sequence [p12(+)] and antisense p12^I sequence [p12(−)], respectively, from the pME-p12 plasmid. All plasmids were confirmed to be in frame by Sanger sequencing. The NFAT luciferase construct pNFAT-Luc contains a trimerized human distal IL-2 NFAT site inserted into the minimal IL-2 promoter and was a gift from G. Crabtree (Stanford University). The AP-1-Luc reporter plasmid was purchased from Stratagene. pCMV-SPORT-β-gal (Gibco) was used as transfection efficiency control.

Pseudotype virus production, concentration and determination of viral titers. To generate vesicular stomatitis virus protein G (VSV-G) pseudotyped lentivirus, 293T cells (4 × 10^6) were seeded into a 10-cm dish and transfected the following day with 2 μg of pHCMV-G (53), 10 μg of pCMVΔR8.2 (53), and 10 μg of pHR′CMV/p12(+)/eGFP or pHR′CMV/p12(−)/eGFP by the calcium phosphate method (52, 53). Supernatant from 10 to 20 dishes was collected at 24, 48, and 72 h posttransfection, cleared of cellular debris by centrifugation at 3,000 rpm for 10 min at room temperature, and then filtered through a 0.2-μm (pore-size) filter. The resulted supernatant was further subjected to centrifugation at

6,500 × g for 16 h at 4°C. The viral pellet was suspended in 300 μl of complete DMEM overnight at 4°C, and the concentrated virus was stored at −80°C.

To determine the virus titer, virus stocks diluted 1:100, 1:1,000, or 1:10,000 were used to infect late-passage 293T cells, and enhanced green fluorescent protein (eGFP) expression was measured by flow cytometry at 48 h postinfection. Briefly, on the day before infection, 10^5 293T cells were seeded into a six-well plate. The medium was removed the following day, and the cells were then incubated with the diluted virus containing 8 μg of Polybrene/ml (Sigma, St. Louis, Mo.). Cells were spin infected by centrifugation at 2,500 rpm for 1 h at room temperature, fed with fresh medium, and cultured for 48 h. The cells were then treated with trypsin (Gibco), pelleted, and resuspended in D-PBS (Gibco) for fluorescence-activated cell sorting (FACS) analysis on an ELITE ESP flow cytometer (Beckman Coulter, Miami, Fla.). HIV *gag* p24 was also measured in an antigen capture assay (Beckman Coulter) to confirm the relative viral titer in the virus stock.

Jurkat T cells and PBMC infection. Jurkat T cells were incubated with VSV-G pseudotyped virus at a multiplicity of infection of 4 in the presence of 8 μg of Polybrene/ml and then spin infected at 2,500 rpm for 2 h at room temperature. The cells were left in the virus-containing media for 16 h, refed with fresh medium, and cultured for 4 days to analyze the IL-2 production in supernatant. PBMC used for infection were preactivated for 3 days with 2 μg of phytohemagglutinin (PHA)/ml in cRPMI. PBMC were incubated with concentrated VSV-G pseudotyped virus at a multiplicity of infection of 30 in the presence of 8 μg of Polybrene/ml and then spin infected at 2,500 rpm for 1 h at room temperature. The cells were left in the virus-containing medium for 16 h, refed, and cultured for 6 days in the absence of PHA. The IL-2 production was measured at day 6 as described in the following sections.

Cell stimulation and IL-2 assay. To test the IL-2 production from Jurkat T cells or PBMC with various stimulation protocols, 1 μg of mouse anti-human CD3 monoclonal antibody (clone HIT3a; Pharmingen, San Diego, Calif.), with or without 1 μg of mouse anti-human CD28 monoclonal antibody (clone Cd28.2; Pharmingen), was immobilized on an enzyme immunoassay-radioimmunoassay 96-well plate (catalog number 3590; Costar) in binding buffer (0.2 M sodium bicarbonate [pH 8.0]) overnight at 4°C. Wells were washed three times with D-PBS, followed by the addition of 10^5 Jurkat T cells or PBMC to wells containing 20 ng of phorbol 12-myristate 13-acetate (PMA; Sigma)/ml or 20 ng of PMA/ml plus 2 μM ionomycin (Sigma) or to wells immobilized with anti-CD3 with or without anti-CD28. After 24 h, supernatant were examined for IL-2 concentration by a standard enzyme-linked immunosorbent assay (ELISA) technique (R&D Systems, Minneapolis, Minn.). An aliquot of the cells was stained with phycoerythrin-conjugated monoclonal anti-CD25 antibody (Pharmingen) and propidium iodide (Molecular Probes, Eugene, Oreg.) to analyze the cell surface CD25 in viable cells by FACS analysis. GFP expression was concurrently analyzed by FACS analysis. The remaining cells were lysed in radioimmunoprecipitation assay buffer as previously described (12), followed by immunoblot assay to test for the expression of p12^I.

To test PBMC proliferation in response to IL-2, PBMC were stimulated with in the presence of similar concentrations of IL-2 measured from p12^I-expressing PBMC. PBMC prepared in an identical manner as our vector trials were cultured in the presence of 0, 100, 200, 500, 1,000, and 2,000 pg of natural human IL-2 (Roche Diagnostic Corp., Indianapolis, Ind. [10,000 U = 5 μg])/ml. Cells were tested for proliferation as previously described (8) by using a tetrazolium dye-based method (CellTiter 96 Cell Proliferation Assay; Promega, Madison, Wis.).

To test the effect of calcium chelator, 10 μM BAPTA-AM {[glycine, $N,N′$,1,2-ethanediylbis(oxy-2,1-phenylene)-bis-N-2-(acetyloxy)methoxy-2-oxoethyl]-[bis (acetyloxy)methyl ester]; Molecular Probes}, 200 nM cyclosporine (Sigma) was added to the infected Jurkat T cells at 37°C 30 min before the cells were seeded into a 96-well plate for different stimulations, followed by 24-h incubation and IL-2 assay. Datum points were expressed as the mean value of IL-2 production from triplicate experiments. Statistical analysis to compare IL-2 production in different samples was performed by using the Student t test.

Transfection and luciferase assay. To test for NFAT or AP-1 transcriptional activity, ca. 10^6 infected Jurkat T cells were electroporated in cRPMI (250 V, 950 μF; Bio-Rad Gene Pulser II) with 5 μg of pNFAT-luc or AP-1-Luc and 0.25 μg of pCMV-SPORT-β-gal plasmid. Transfected cells were seeded in 24-well plates at a density of 2 × 10^5/0.5 ml and were stimulated with 20 ng of PMA/ml in the presence or absence of 2 μM ionomycin or anti-CD3 and/or anti-CD28 monoclonal antibody (each at 5 μg/ml) at 6 h posttransfection, followed by an 18-h incubation prior to lysis for analysis of luciferase activity (Promega) as previously described (12).

To test the NFAT transcriptional activities in Jurkat T cells transfected with different p12^I truncation mutants and p12^I point mutants, 10^7 cells were electroprated in cRPMI (350 V, 975 μF; Bio-Rad Gene Pulser II) with 30 μg of

expression plasmid, 10 μg of pNFAT-luc, and 1 μg of pCMV•SPORT-β-gal plasmid to normalize for transfection efficiency. Transfected cells were seeded in six-well plates at a density of 5 × 10^5/ml and were stimulated with 20 ng of PMA/ml at 6 h posttransfection, followed by an 18-h incubation and analysis for luciferase activities.

For investigation of the effect of pharmacological inhibitors, 200 nM cyclosporine, 10 μM BAPTA-AM, or 10 μM MEK-1 inhibitor U0126 [1,4-diamino-2,3-dicyano-1,4-bis(2-aminophenylthio)butadiene (Promega)] was added to transfected cells 6 h posttransfection for 30 min at 37°C, followed by stimulation with PMA and luciferase analysis.

Immunoblot assay. The expression of p12^I, p12^I truncation, and point mutants was analyzed by immunoblot assay. Briefly, infected Jurkat T cells or transfected 293T cells were lysed in radioimmunoprecipitation assay buffer (150 mM NaCl, 50 mM Tris [pH 8.0], 10 mM EDTA, 10 mM NaF, 10 mM Na$_4$P$_2$O$_7$ · H$_2$O, 1% NP-40, 0.5% deoxycholic acid, 0.1% sodium dodecyl sulfate [SDS], 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, and Complete protease inhibitor [Roche]), and the cell lysates were cleared by centrifugation. A total of 50 μg of 293T cell lysates or 300 μg of infected Jurkat T-cell lysates were separated by SDS–15% polyacrylamide gel electrophoresis, followed by transfer to nitrocellulose membranes. Membranes were blocked with 5% milk for 2 h, incubated with mouse anti-HA monoclonal antibody (clone 16B-12; Covance, Richmond, Calif.) overnight at 4°C and developed by using horseradish peroxidase-labeled secondary antibody and enhanced chemiluminescence (Cell Signaling Technologies, Beverly, Mass.).

Indirect immunofluorescence assay. For visualization, the intracellular localization of mutant p12^{I}15-47 and p12^{I}15-47KKLL, HeLa-Tat cells were seeded into LAB-TAK chamber slides (Nalgene Nunc International, Rochester, N.Y.) and were transfected with 4 μg of p12^{I}15-47 or p12^{I}15-47KKLL plasmid by using Lipofectamine Plus (Invitrogen, Carlsbad, Calif.). Cells were fixed for 15 min in 4% paraformaldehyde at 24 h posttransfection, followed by incubation with primary antibodies: mouse anti-HA monoclonal antibody and rabbit anti-calreticulin polyclonal antibody (Affinity Bioreagents, Golden, Colo.) in antibody dilution buffer (10 mM NaPO$_4$, 0.5 M NaCl, 0.5% Triton X-100, 2% bovine serum albumin, 5% normal goat serum) for 1 h at room temperature. After three washes, cells were incubated with indocarbo-cyanin 3 (Cy3)-labeled goat antimouse (Jackson Immunogen, West Grove, Pa.) and Alexa Flour 488-labeled goat anti-rabbit (Molecular Probes) in antibody dilution buffer for 45 min before a final wash and mounting in glycerol. Cell nuclei were stained with bisbenzimide H33528 (Hoechst 33528; Calbiochem, San Diego, Calif.). Fluorescence microscopy and image collection was performed by using a Zeiss Axioplan2 fluorescence microscope (Carl Zeiss Optical, Chester, Va.) and a SPOT camera (model 1.4.0; Diagnostic Instruments, Inc., Sterling Heights, Mich.) with Adobe Photoshop 5.0 software.

RESULTS

p12^I expression from lentivirus vector in Jurkat T cells. Vector pHR′CMV/p12(+)/eGFP or pHR′CMV/p12(−)/eGFP was constructed to allow p12^I and eGFP to be coexpressed or to allow eGFP to be expressed alone from a polycistronic mRNA initiated at an internal cytomegalovirus (CMV) immediate-early promoter (Fig. 1A). Expression of the eGFP gene was initiated by an internal ribosome entry site element that allowed the efficient translation of downstream sequences. Jurkat T cells were transduced with the vector pHR′CMV/p12(−)/eGFP or pHR′CMV/p12(+)/eGFP, and flow cytometric analysis and immunoblot assay at day 4 postinfection demonstrated the expression of GFP and p12^I protein (Fig. 1B). The unactivated Jurkat T-cell line did not express the IL-2R α (CD25), and expression of p12^I did not enhance CD25 expression (data not shown).

p12^I expression increases IL-2 production by T-cell antigen receptor ligation in the presence or absence of CD28 costimulation. HTLV-1 p12^I expression in Jurkat T cells enhances basal intracellular calcium (12) and selectively activates transcription factor NFAT (2). Since IL-2 is a downstream gene of NFAT activation and the secretion of IL-2 is an indicator of T-cell activation, we tested the IL-2 production in Jurkat T-cell

supernatants after costimulation of cells with various agents that stimulate T-cell activation (Fig. 2). Expression of p12^I in Jurkat T cells displayed a significant 1.5- to 2-fold enhancement of IL-2 production when cells were stimulated with anti-human CD3 alone or combined with anti-human CD28 (Fig. 2A and B). Similar enhanced IL-2 production has been demonstrated in Jurkat T cells expressing HIV Nef (46, 50). However, p12^I had no effect on IL-2 production from cells stimulated with combination of PMA plus ionomycin (Fig. 2C). These data suggested that p12^I facilitates T-cell activation induced by T-cell receptor ligation, and this activation is probably overriden by the downstream potent stimulations with PMA plus ionomycin.

Unstimulated Jurkat T cells did not produce IL-2, and PMA or ionomycin stimulation alone did not elicit production of IL-2 in the presence or absence of p12^I (data not shown). As expected, upon anti-CD3 and anti-CD28 stimulation, the Jurkat T-cell surface level of CD25 increased. However, expression of p12^I did not enhance further CD25 expression during cell surface receptor stimulation (data not shown). These findings were similar to reports of HIV Nef on T-cell activation, in which Nef expression in Jurkat T cells did not alter cell surface CD25 expression in the presence of T-cell receptor ligation but did enhance IL-2 production (46, 50).

Enhanced IL-2 production mediated by p12^I expression is calcium pathway dependent. p12^I localizes in the ER and cis-Golgi compartments (13) and increases basal intracellular calcium, possibly by facilitating calcium release from ER stores and subsequently enhancing extracellular calcium influx (12). The expression of p12^I increased IL-2 production in cells stimulated by T-cell receptor ligation, but p12^I expression had no effect on IL-2 production when cells were potently stimulated by PMA plus the calcium ionophore, ionomycin. Taken together, these findings suggest that the enhanced IL-2 production mediated by p12^I is calcium dependent, and this effect is overcome by large intracellular calcium increases induced by ionomycin. To test this hypothesis, Jurkat T cells transduced by pHR′CMV/p12(+)/eGFP were treated with calcium chelator, BAPTA-AM, before the T-cell receptor ligation mimicked by anti-CD3 stimulation alone or in combination with anti-CD28 costimulation. Without the addition of BAPTA-AM, p12^I expression significantly enhanced IL-2 production compared to p12(AS)-transduced Jurkat T cells (Fig. 3). BAPTA-AM treatment was capable of reducing the IL-2 production in both p12^I- and p12(AS)-transduced Jurkat T cells. Importantly, intracellular calcium chelation inhibited the enhanced IL-2 production mediated by p12^I expression in the presence of anti-CD3 alone (Fig. 3A) or with anti-CD28 (Fig. 3B), indicating that p12^I enhances IL-2 production in a calcium-dependent manner.

To further confirm that expression of p12^I enhances IL-2 production through a calcium-dependent pathway, we measured IL-2 secretion in the presence or absence of calcineurin inhibitor, cyclosporine. Calcineurin is a distal phosphatase that is activated by increased intracellular calcium after T-cell receptor ligation. Activated calcineurin dephosphorylates NFAT and thereby induces downstream gene expression. cyclosporine completely abolished the increase in IL-2 production in cells expressing p12^I and p12(AS) in the presence of anti-CD3 with or without anti-CD28 (Fig. 3). Therefore, p12^I appears to act upstream of calcineurin via the calcium-dependent pathway.
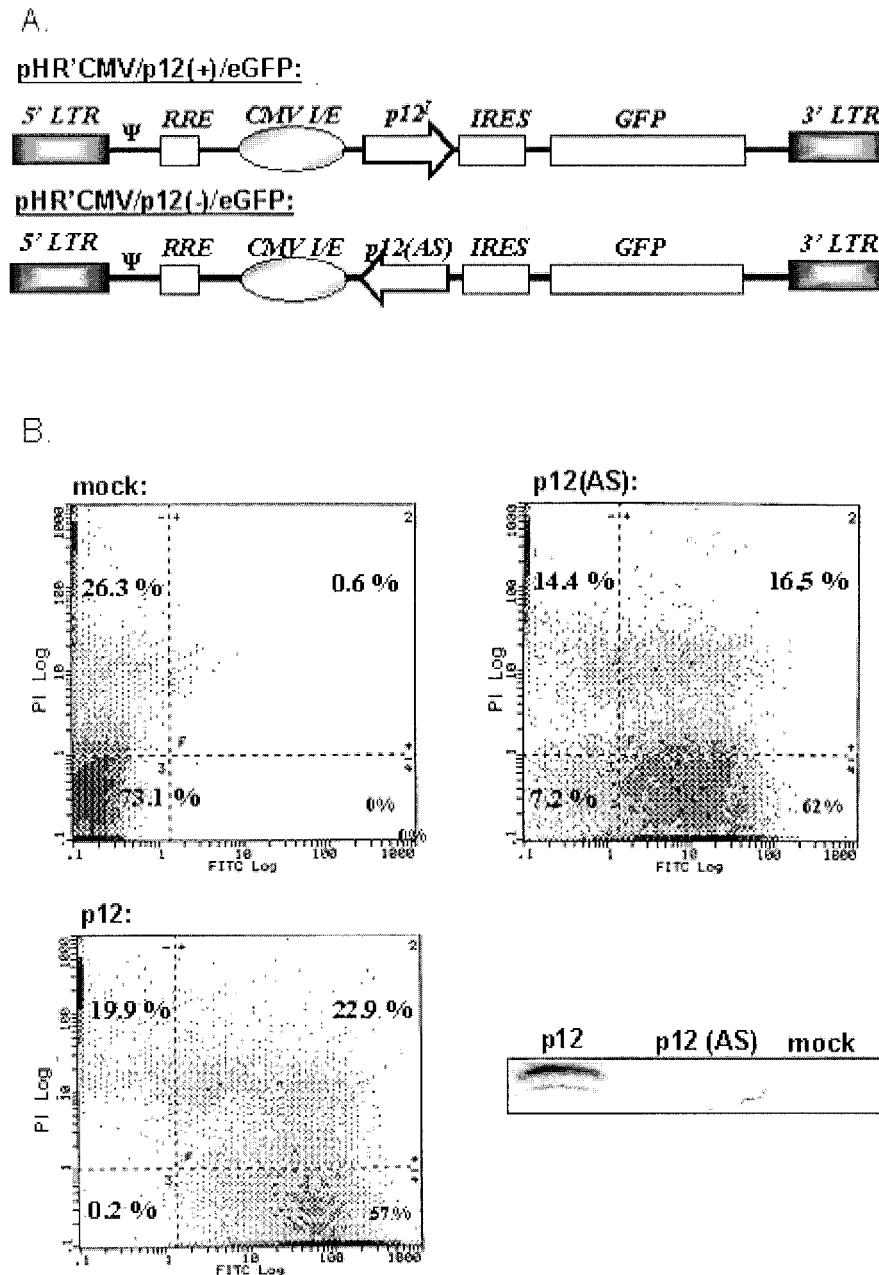
FIG. 1. p12[I] expression in Jurkat T cells. (A) Schematic representation of the p12 transgene plasmids pHR′CMV/p12(+)/eGFP and pHR′CMV/p12(−)/eGFP. (B) The HTLV-1 p12[I] gene was introduced into Jurkat T cells by lentiviral transduction as described in Materials and Methods. Cells were transduced with sense p12[I] (p12[I], expressing both p12[I] and eGFP) or antisense p12[I] [p12(AS), expressing eGFP alone] by lentiviral vectors or mock infected. At day 4 posttransduction, an aliquot of cells was stained with propidium iodide (PI) and anti-CD25 antibody by flow cytometry. GFP expression was concurrently analyzed. Approximately 50% of infected cells were found to be GFP positive in both p12[I] and p12(AS) samples. Samples lysed from approximately 2 million cells were tested for p12[I] expression by immunoblot assay. The results were representative of three independent infection experiments.

Expression of p12[I] hyperactivates NFAT transcription in response to cell surface receptor activation. We have reported that p12[I] expression enhances NFAT transcriptional activities in Jurkat T cells (2). To test whether NFAT activation was correlative with the enhanced IL-2 production in p12[I]-expressing cells, transduced Jurkat T cells were transfected with pNFAT-luc plasmid and the transcriptional activities of NFAT were measured after cells were stimulated with various agents.

p12[I] expression enhanced NFAT transcriptional activities six-fold in the presence of anti-CD3 alone and threefold when cells were stimulated with anti-CD3 and anti-CD28 compared to the control cells expressing GFP alone (Fig. 4A). Of note, anti-CD3 and anti-CD28 dual stimulation in Jurkat T cells elicited increased NFAT reporter gene activities compared to anti-CD3 stimulation alone (data not shown). Jurkat T cells that expressed p12[I] exhibited threefold-higher NFAT tran-
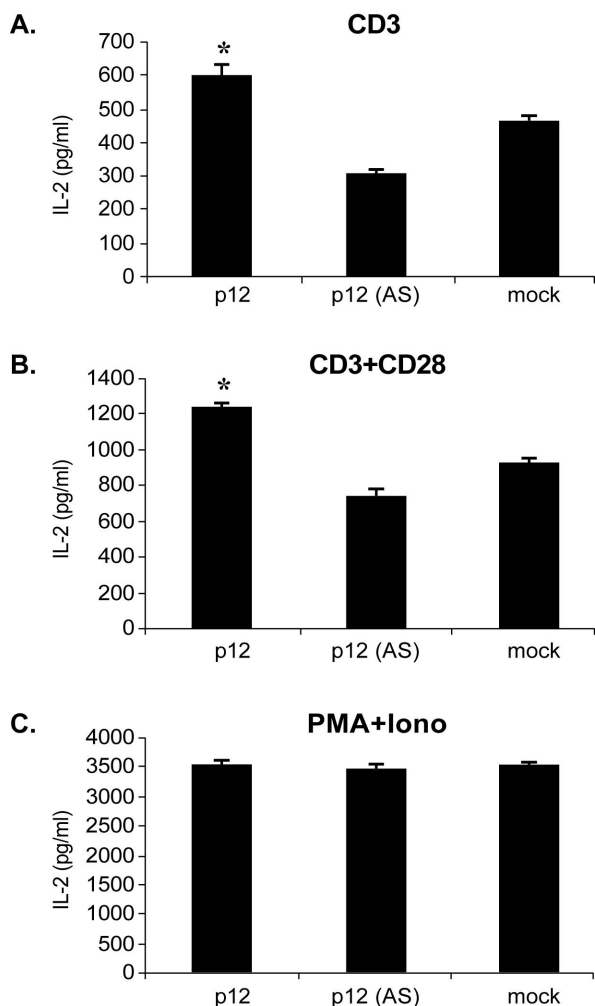
FIG. 2. p12^I increases IL-2 production by T-cell antigen receptor ligation in the presence or absence of CD28 costimulation. Transduced Jurkat T cells were activated by immobilized anti-CD3 (coated at 5 μg/ml) (A), immobilized anti-CD3 plus anti-CD28 (coated at 5 μg/ml) (B), or PMA plus 2 μM ionomycin (Iono) (C) in 96-well plates. After 24 h at 37°C, IL-2 secreted in the supernatant was measured by ELISA techniques. p12^I-transduced Jurkat T cells secreted significantly higher IL-2 than p12(AS)-transduced cells or mock-infected cells in the presence of anti-CD3 with or without anti-CD28 stimulation. ∗, $P < 0.05$. Data represent mean IL-2 concentrations from triplicate wells. The results are representative of three independent infection experiments. Statistical significance was analyzed by using the Student t test.

scription as indicated by enhanced luciferase reporter gene activity in the presence of both anti-CD3 and anti-CD28 stimulation compared to control cells expressing GFP alone. Corroborating our data of IL-2 production, this hyperactivation was not present in unstimulated cells (data not shown) in cells stimulated with PMA plus ionomycin or in cells stimulated with PMA alone. These data indicate that the NFAT activation mediated by p12^I and T-cell receptor ligation likely result in enhanced IL-2 production. Furthermore, the stimulatory effect of p12^I specifically involved NFAT-dependent transcription, since the activity of AP-1 dependent transcription remained unchanged regardless of the stimulation conditions (Fig. 4B).

Expression of p12^I in PBMC enhances the IL-2 production in the presence of PMA stimulation. As an extension of the

above studies in Jurkat T cells, we transduced the HTLV-1 natural target cells, PBMC, and analyzed the effect of p12^I expression on IL-2 production in PBMC. Preactivated PBMC by PHA were transduced with pHR′CMV/p12(+)/eGFP or pHR′CMV/p12(−)/eGFP vector, and GFP expression was analyzed by flow cytometry (Fig. 5A). We observed a higher GFP expression (ca. 50 to 60%) at day 6 posttransduction than at the earlier time points (data not shown). Therefore, the IL-2 production in the supernatant was measured at day 6 posttransduction after the cells were stimulated with various agents. Receptor ligation by anti-CD3 in the presence or absence of anti-CD28 did not significantly enhance IL-2 production in PBMC expressing p12^I compared to the control cells expressing GFP alone (Fig. 5B and C). Expression of p12^I had no effect on IL-2 production when cells were stimulated with
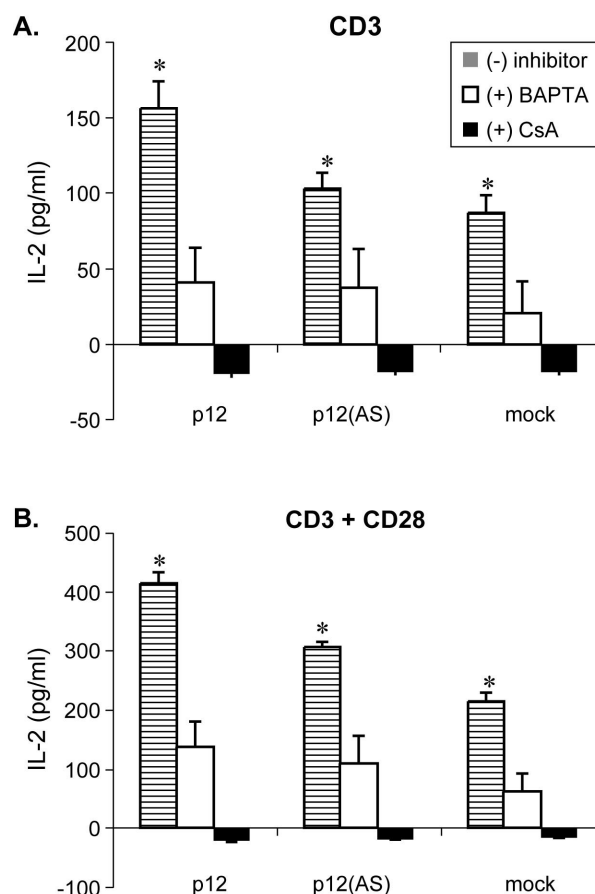

FIG. 3. Enhanced IL-2 production mediated by p12^I is calcium pathway dependent. Transduced Jurkat T cells were treated with BAPTA-AM (BAPTA) or cyclosporine before the T-cell receptor ligation mimicked by anti-CD3 (A) or with anti-CD28 stimulation (B). p12^I-transduced Jurkat T cells secreted significantly higher levels of IL-2 than did p12(AS)-transduced cells or mock-infected cells in the presence of anti-CD3 alone (A) or with anti-CD28 stimulation (B). BAPTA-AM and cyclosporine treatment resulted in significantly reduced IL-2 production in p12^I- and p12(AS)-transduced cells, as well as in mock-infected cells. However, IL-2 production in p12^I-expressing cells after the addition of either inhibitor was not significantly different from that seen in p12(AS)-transduced cells or in mock-infected cells. Values are the means of triplicate or six samples. Statistical significance was analyzed by using the Student t test. ∗, $P < 0.05$.
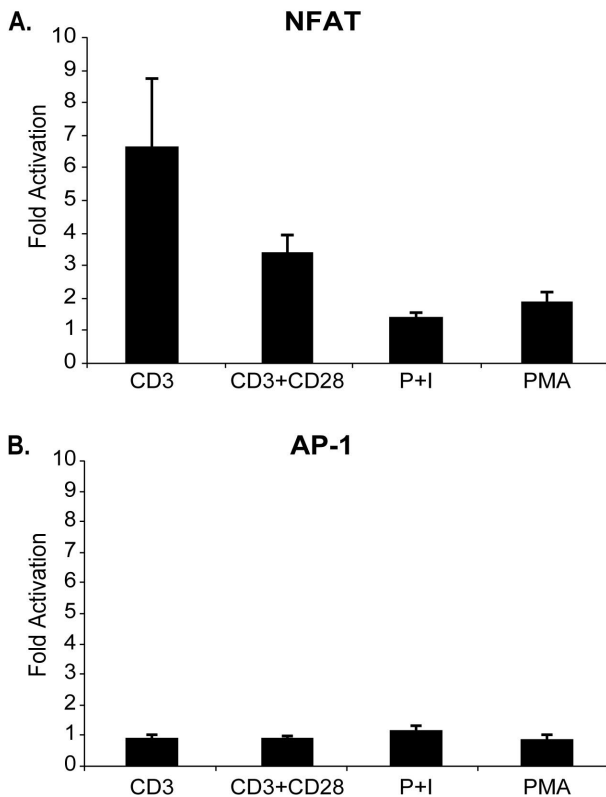
A.

NFAT

B.

AP-1

FIG. 4. p12^I promotes NFAT activation in response to CD3 with or without CD28 stimulation. Transduced Jurkat T cells transfected with pNFAT-Luc (A) or AP-1-Luc (B) plasmid were stimulated with either PMA, PMA plus ionomycin (P+I), anti-CD3, or anti-CD3 plus anti-CD28. The graph depicts the fold induction of NFAT or AP-1 luciferase activity in cells expressing p12^I (p12) versus those transduced with antisense p12 (AS). Datum points are the means of triplicate samples from three independent experiments.

PMA plus ionomycin (Fig. 5D). Interestingly, PBMC expressing p12^I had a sixfold-higher IL-2 concentrations in cell culture supernatants compared to control cells after stimulation with PMA alone (Fig. 5E). These data likely represent the differential response of primary PBMC compared to the transformed Jurkat T-cell line (39).

To address the issue of differential dose response with anti-CD3 and anti-CD28 in PBMC, we tested the IL-2 production from PBMC with various amounts of anti-CD3 (0.06, 0.2, 0.5, and 1.0 μg), with or without 1 μg of anti-CD28 (optimal response dose to costimulate PBMC). We found differences of <10% in IL-2 production between these various stimulation protocols, indicating that the differential response between Jurkat T cells and PBMC was not due to saturating concentrations of the stimuli for PBMC (data not shown).

We have previously demonstrated IL-2 responsiveness of HTLV-1 immortalized T-cell lines (8). To test whether the quantities of IL-2 elicited from p12^I-expressing PBMC could stimulate normal PBMC to proliferate, we analyzed cell proliferation of PBMC in the presence of similar concentrations of IL-2 measured from p12^I-expressing PBMC. PBMC prepared in a manner identical to that used in our vector trials were cultured in the presence of 0, 100, 200, 500, 1,000, and 2,000 pg of recombinant human IL-2/ml. At concentrations similar to

the levels of IL-2 produced from p12^I-expressing PBMC, cell proliferation was measurably increased (25% increase in optical density in proliferation assay) after as little as 4 h of incubation (Fig. 6). At this rate of proliferation, PBMC would be predicted to double in number in approximately 16 h. Thus, the quantity of IL-2 induced by p12^I-expressing PBMC would be predicted to have a functional influence on lymphocyte proliferation.

Identification of two positive (amino acids [aa] 33 to 47 and 87 to 99) and two negative regions (aa 1 to 14 and 70 to 86) in p12^I for NFAT activation. To investigate the possible domains in p12^I required for NFAT activation, Jurkat T cells were transiently cotransfected with full-length or p12^I truncation mutants in combination of pNFAT-luc plasmid, and the transcriptional activities of NFAT were analyzed. The expressions of full-length and truncated p12^I were tested by immunoblot assay (Fig. 7A). The majority of p12^I truncation mutants expressed at equivalent levels compared to full-length protein. Mutant p12^I 15-99 expression induced higher NFAT luciferase activity than full-length protein (Fig. 7B), suggesting the presence of an inhibitory region in the amino-terminal 14 aa (first negative region). Further amino-terminal deletion displayed a NFAT luciferase activity similar to full-length p12^I in cells transfected with mutant p12^I 32-99. However, expression of mutant p12^I 48-99 resulted in a reduced NFAT transcriptional activity (Fig. 7B), indicating the region aa 33 to 47 contains a positive domain (first positive region). While the N-terminal half of p12^I contained one negative region (aa 1 to 14) and one positive region (aa 33 to 47) for NFAT activation, analysis of C-terminal mutants revealed another positive and another negative region. Carboxy-terminal deletion of 13 aa in mutant p12^I 1-86 resulted in a dramatic reduction of NFAT activity (Fig. 7B). Thus, aa 87 to 99 likely contains the second positive region for NFAT activation. To our surprise, further C-terminal deletion in mutant p12^I 1-47 almost restored the NFAT activation mediated by full-length protein, implying the presence of another inhibitory domain in region aa 48 to 86 (second negative region). Analysis of mutants containing both amino- and carboxy-terminal deletions further confirmed that the aa 1 to 15 is a negative region since a higher NFAT activity was detected in cells expressing p12^I 15-86 compared to cells expressing 1-86. Mutant p12^I 15-69 expression elicited a twofold-higher NFAT luciferase activity than did the full-length protein (Fig. 7B), indicating the presence of the second negative region in aa 70 to 86. Of note, all mutants except p12^I 15-47 localized in the ER perinuclear region. Therefore, the differential NFAT activations in cells expressing individual mutants are likely due to the deleted sequence. The NFAT transcriptional activities mediated by all p12^I truncation mutants, except 32-99, were significantly different ($P < 0.05$) from those of wild-type p12^I. However, we cannot exclude the possibility that alteration of the p12^I protein structure is responsible for differential NFAT activations. Expression of mutant p12^I 15-47, which localizes in both the nucleus and the cytoplasm, failed to enhance NFAT luciferase activity (Fig. 7B), suggesting the ER localization is necessary for p12^I mediated NFAT activation.

To test whether NFAT transcriptional activation induced by individual mutants was calcium and Ras/MAPK pathway dependent, a number of inhibitors, including BAPTA-AM, cyclosporine, and an inhibitor of MAP kinase kinase (MEK-1),
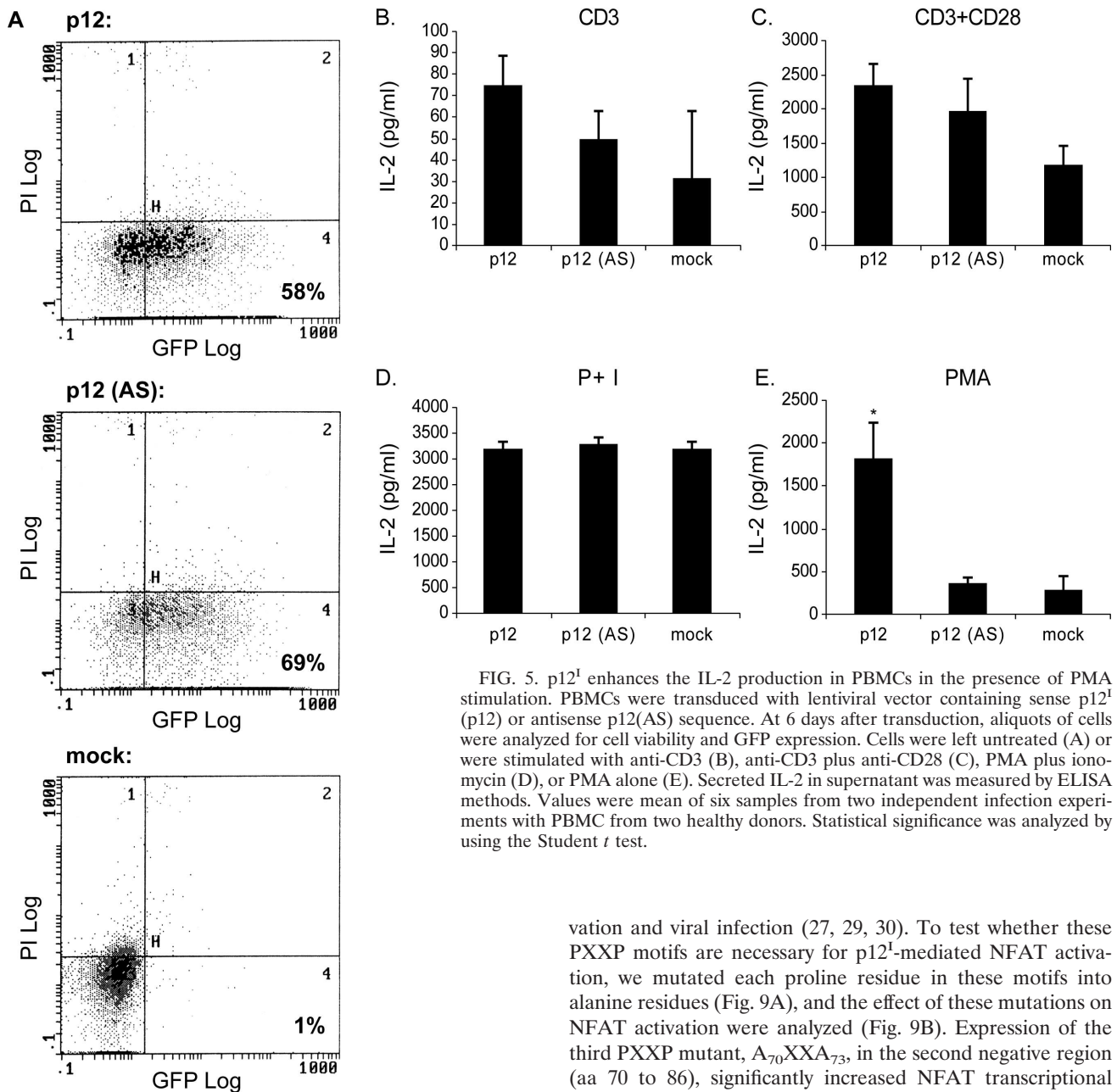
FIG. 5. p12^I enhances the IL-2 production in PBMCs in the presence of PMA stimulation. PBMCs were transduced with lentiviral vector containing sense p12^I (p12) or antisense p12(AS) sequence. At 6 days after transduction, aliquots of cells were analyzed for cell viability and GFP expression. Cells were left untreated (A) or were stimulated with anti-CD3 (B), anti-CD3 plus anti-CD28 (C), PMA plus iono-mycin (D), or PMA alone (E). Secreted IL-2 in supernatant was measured by ELISA methods. Values were mean of six samples from two independent infection experiments with PBMC from two healthy donors. Statistical significance was analyzed by using the Student t test.

U0126, were used to treat the transfected Jurkat T cells before the addition of PMA. All three inhibitors tested completely abolished the NFAT activation mediated by two highly active mutants, p12^I 15-99 and p12^I 15-69 (Fig. 8), indicating these mutants activate NFAT in a similar calcium- and Ras/MAPK-dependent manner as full-length protein.

The third SH3-binding domain (aa 70 to 73) is responsible for the negative effect of region aa 70 to 86 on NFAT activation. The two positive and two negative regions we identified for NFAT activation contain individual SH3-binding domains, the PXXP motif. The SH3-binding domain has been demonstrated to be involved in protein-protein interaction, and the PXXP motif in HIV Nef is necessary for Nef-mediated NFAT acti-

vation and viral infection (27, 29, 30). To test whether these PXXP motifs are necessary for p12^I-mediated NFAT activation, we mutated each proline residue in these motifs into alanine residues (Fig. 9A), and the effect of these mutations on NFAT activation were analyzed (Fig. 9B). Expression of the third PXXP mutant, $A_{70}XXA_{73}$, in the second negative region (aa 70 to 86), significantly increased NFAT transcriptional activity (190%) compared to wild-type p12^I. Thus, in parallel to our data with serial deletion mutants, the third PXXP motif appears to mediate the negative effect of region aa 70 to 86 on NFAT activation. Expression of the first PXXP mutant (A_8XXA_{11}) and second PXXP mutant ($A_{35}XXA_{38}$) minimally enhanced and decreased NFAT activation, respectively. The last PXXP mutant ($A_{90}XXA_{93}$), in the second positive region (aa 86 to 99), enhanced the NFAT activation. The functional significance of specific residues within the PXXP motifs of p12^I in NFAT activation remains to be further clarified.

ER localization is required for p12^I-mediated NFAT activation. Although the mutant p12^I 15-47 contains a positive region (aa 33 to 47), expression of this mutant displayed a dramatically reduced NFAT transcriptional activity. In addition, this mutant fails to maintain ER localization; instead, it mainly accumulates in the nucleus (13). To test whether ER local-
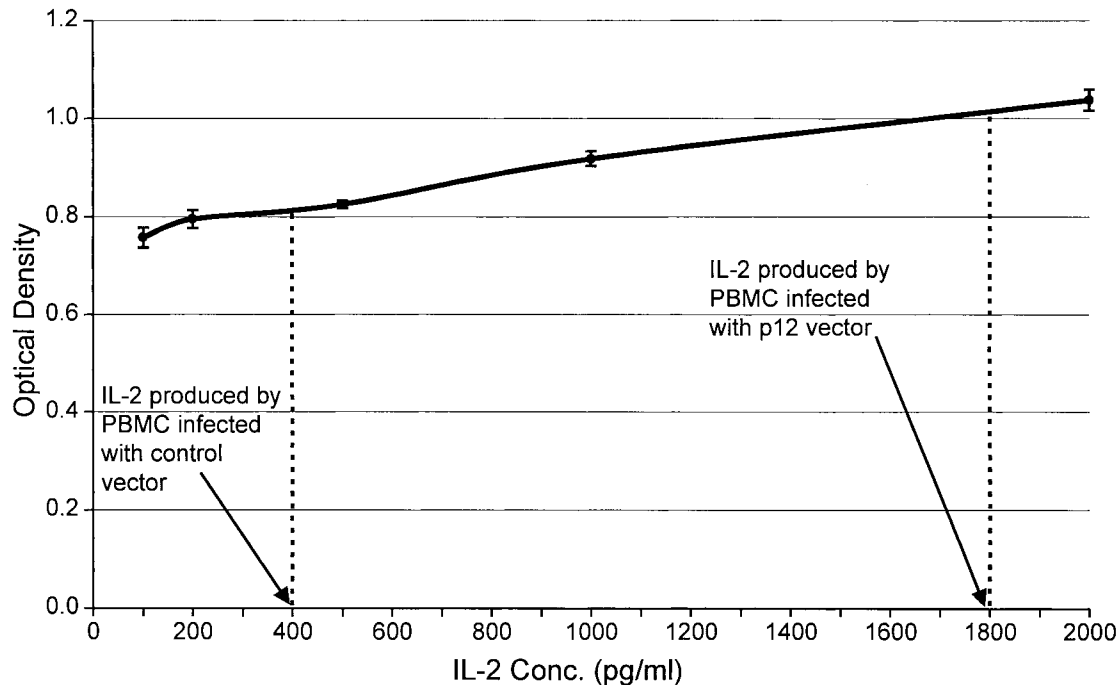
FIG. 6. To test PBMC proliferation in response to IL-2, PBMC were stimulated with in the presence of similar concentrations of IL-2 measured from p12[I]-expressing PBMC. PBMC prepared in a manner identical to that used in our vector trials were cultured in the presence of 0, 100, 200, 500, 1,000, or 2,000 pg of natural human IL-2 (10,000 U = 5 μg [Roche])/ml. Cells were tested for proliferation as previously described (8) by using a tetrazolium dye-based method (CellTiter 96 cell proliferation assay [Promega]). Linear analysis was performed to show the relationship between IL-2 concentration and PBMC proliferation. The range of IL-2 concentrations from PBMC infected with control or p12-expressing vectors is indicated on the graph.

ization is necessary for p12[I]-mediated NFAT activation, we tagged the C terminus of this mutant with the sequence of a well-recognized ER targeting signal, KKLL (20, 43), and analyzed the location of the new protein 15-47KKLL, as well as NFAT activation in Jurkat T cells expressing this chimera. As expected, the chimera 15-47KKLL accumulated in the perinuclear region and colocalized with calreticulin, an ER luminal protein, in transfected HeLa-Tat cells (Fig. 10A). Expression of this chimera in Jurkat T cells partially restored the NFAT activation compared to the low NFAT activity in cells expressing 15-47 (Fig. 10B). Thus, ER localization is required for p12[I]-mediated NFAT activation. The chimera protein, which regained the ER localization, only restored half of the NFAT activation induced by full-length p12[I], suggesting that the first positive region (aa 33 to 47) is not sufficient for full activation of NFAT.

DISCUSSION

In this study, we used a lentiviral system to express p12[I] protein in transduced Jurkat T cells and in primary human PBMC. Under the condition of T-cell receptor ligation and anti-CD28 costimulation, expression of p12[I] enhanced IL-2 production in Jurkat T cells. As a downstream gene responsive to activation of multiple signal pathways in T cells, IL-2 is an indicator for T-cell activation. Activation of T lymphocytes results in cell division, which is a prerequisite for the proviral DNA of retrovirus, including HTLV-1, to integrate into the host cell genome and permit the subsequent viral replication

and productive infection. Thus, p12[I] expression appears to augment T-cell activation to facilitate the viral replication and productive infection. This finding provides a mechanism for HTLV-1 to establish infection in resting T cells (1) and also is supportive of our previous finding that selective ablation of mRNA containing p12[I]-coding sequence reduces viral infectivity in a rabbit model of infection (9). Consistent with our data presented here, T cells would become hypersensitive to T-cell activation signals when HTLV-1 p12[I] is present and become highly permissive for subsequent viral infection.

p12[I] localizes in the ER and cis-Golgi compartments and associates with the calcium-binding protein, calreticulin (13). p12[I] expression in Jurkat T cells increases the level of intracellular calcium (12) and selectively activates NFAT (2). Therefore, the elevated IL-2 secretion in p12[I]-expressing T cells likely results from the activation of calcium-dependent pathway. Indeed, the calcium chelator, BAPTA-AM and the calcineurin inhibitor, cyclosporine, inhibited the enhanced IL-2 production in cells expressing p12[I]. In addition, expression of p12[I] activated the NFAT transcriptional activity in the presence of surface receptor CD3 and CD28 stimulation but not in the presence of PMA stimulation. These results are in contrast to our previous finding in which we transiently overexpressed p12[I] in Jurkat T cells and reported the selective activation of NFAT in the presence of PMA. This discrepancy is likely related to the levels of p12[I] expression with each model system. We observed much higher p12[I] expression in transient transfected Jurkat T cells compared to transduction of p12[I]

an approximately sixfold higher level of IL-2 in the presence of PMA. However, p12^I expression in PBMC did not significantly enhance IL-2 secretion during T-cell-receptor stimulation. These data may represent the differential responses in primary
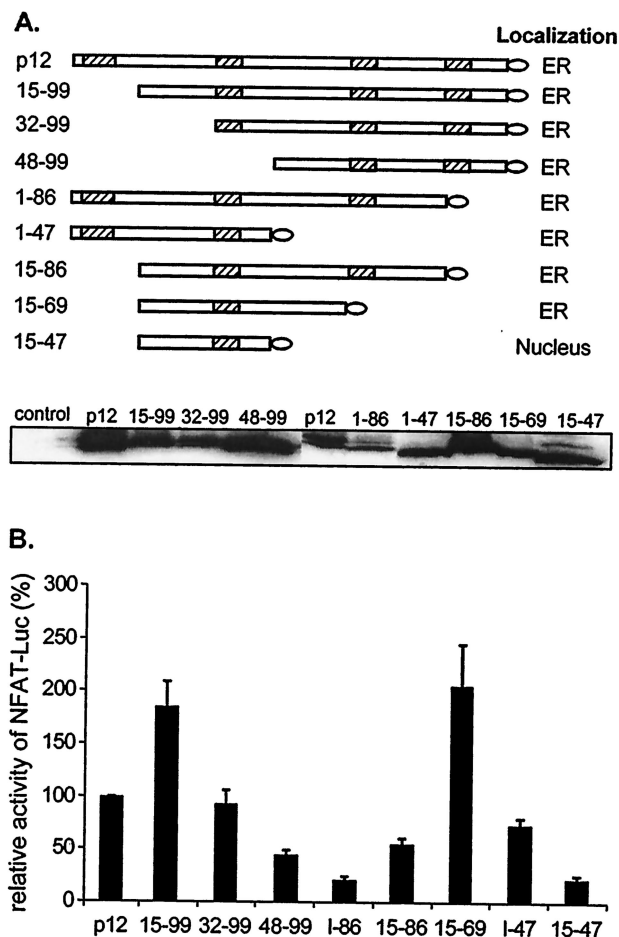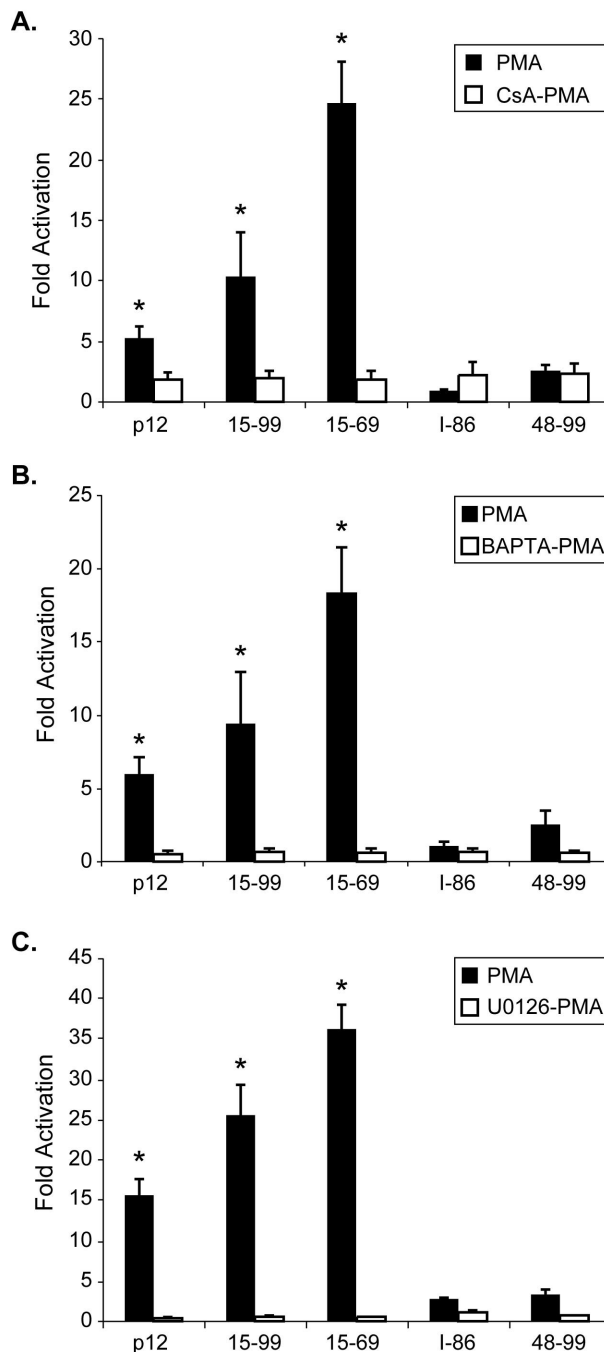

FIG. 7. Two positive regions (aa 33 to 47 and 87 to 99) and two negative regions (aa 1 to 14 and 70 to 86) are identified in p12^I for NFAT activation. (A) Schematic representation and expression of wild-type p12^I and serial truncation mutants. The hatched box indicates the PXXP motif, and the circle indicates the HA tag. 293T cells were transfected with pMEp12 or mutant plasmids. The expression of wild-type p12^I and mutants was tested by immunoblot assay at 48 h posttransfection. (B) Jurkat T cells were electroporated with pMEp12 or plasmids expressing truncation mutants in combination with pNFAT-Luc reporter plasmid, and the NFAT luciferase activities were determined 18 h after PMA treatment. The NFAT transcriptional activities mediated by all p12^I truncation mutants, except for 32-99, were significantly different ($P < 0.05$) from that of wild-type p12^I. The data are presented as relative NFAT luciferase activities compared to wild-type p12^I. The values are the mean of four independent transfection experiments. Statistical significance was analyzed by using the Student t test.

FIG. 8. Hyperactivated p12^I truncation mutants induce NFAT activation in the same fashion as wild-type p12^I. Jurkat T cells were transfected with plasmids containing different mutants in combination with pNFAT-Luc plasmid, and cells were treated with cyclosporine (A), BAPTA-AM (B), or U0126 (C) for 30 min before PMA was added. These inhibitors significantly reduced NFAT activation mediated by the wild type and the hyperactivated mutants 15-99 and 15-69. Datum points are the means of three independent transfection experiments. Statistical significance was analyzed by using the Student t test.
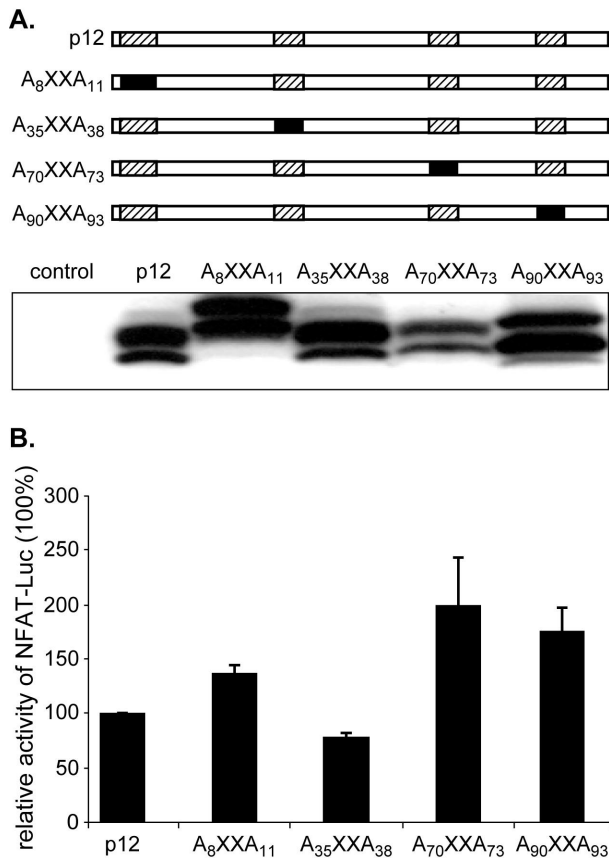
using the lentiviral vector. Therefore, the relatively lower levels of p12^I expression by an internal CMV promoter via the lentiviral system may not be enough to trigger the NFAT activation and IL-2 production in the presence of PMA alone. However, when relative strong stimulations, such as surface receptor ligation, are provided, low amounts of p12^I expression can facilitate the Jurkat T-cell activation. The production of HTLV-1 viral transcripts is very low, and the viral proteins are not easily detectable in naturally infected PBMC (16, 18, 41). Thus, the low levels of p12^I expression via the lentiviral system may be more biologically representative of natural infection. In contrast to Jurkat T cells, expression of p12^I in PBMC induced

A.

B.

FIG. 9. The mutant $A_{70}XXA_{73}$ significantly increases NFAT activity. (A) Schematic representation and expression of wild-type $p12^I$ and point mutants. The hatched box indicates the PXXP motif, and the black box indicates the mutated PXXP motif. (B) Jurkat T cells were transfected with $p12^I$ point mutants that replaced proline with alanine in PXXP motifs and pNFAT-Luc plasmid, followed by 18 h of PMA treatment and measurement of luciferase activities. Data are presented as relative NFAT luciferase activities compared to wild-type $p12^I$. The NFAT transcriptional activities mediated by all $p12^I$ point mutants were significantly different ($P < 0.05$) from wild-type $p12^I$. Values are means of at least three independent transfection experiments. Statistical significance was analyzed by using the Student t test.

T lymphocytes compared to transformed T cells. Our group has observed the prolonged CREB phosphorylation and increased basal HTLV-1 transcription in PBMC after mitogen stimulation compared to Jurkat T cells (39). Therefore, primary T lymphocytes may contain a relative intact cell signal machinery, which is more sensitive in response to activation signals after PMA stimulation, such as protein kinase C activation. Expression of $p12^I$ had no effect on IL-2 production and NFAT activation when downstream strong stimulations, i.e., with PMA plus ionomycin, are provided, indicating that the function of $p12^I$ is calcium dependent and that ionomycin stimulation overrides the effect of $p12^I$ on regulation of calcium homeostasis.

The role of the IL-2/IL-2R signaling pathway in HTLV-1 early infection, virus-induced immortalization, and the transformation process is not clear. Earlier studies (47, 48, 51) demonstrated that Tax expression induces the activation of IL-2R α and IL-2 genes. These activation events mediated by

Tax presumably occur via NF-κB activation and are cyclosporine resistant. However, the temporal expression pattern of viral *tax* mRNA is not consistent with the pattern of IL-2 mRNA in HTLV-1-infected primary lymphocytes. IL-2 is transiently expressed during the early phase of the infection when viral integration is polyclonal after HTLV-1 infection of human primary lymphocytes. It is undetectable at a later stage when viral integration is oligoclonal (23). In contrast, *tax* mRNA is scarcely expressed in the polyclonal phase but is abundantly expressed in the oligoclonal stage. Therefore, additional viral protein expressed in the early phase of viral infection may induce the IL-2 production in the initial phase. The quantities of IL-2 elicited from $p12^I$-expressing PBMC were able to elicit proliferation PBMC and therefore would be predicted to have a functional influence on lymphocyte proliferation. Our findings support the idea that $p12^I$ may trigger early IL-2 expression to facilitate HTLV-1 infection.

A similar viral protein, Nef, modulates calcium signaling through interaction with IP3 receptor (31) and thus activates NFAT (28), as well as enhances IL-2 production in T lymphocytes (46, 50). Nef has been demonstrated to associate with HIV virion (26, 56) and is selectively expressed before the integration to modulate the resting T-cell activity (54). Therefore, it will be important to determine whether $p12^I$ is a virion-associated protein or whether $p12^I$ mRNA and protein are selectively expressed before integration.

HTLV-1-transformed T cells display constitutive tyrosine phosphorylation of IL-2R-coupled signaling proteins, including Jak1, Jak3, Stat3, and Stat5 (33, 34, 36, 55). Expression of $p12^I$ and signaling molecules involved in the IL-2R signaling pathway in 293T cells induced the increased DNA-binding activity of Stat5 (40). The mechanism of $p12^I$-mediated Stat5 activation may be related to the interaction between IL-2R (β and γ) and $p12^I$. Alternatively, expression of $p12^I$ may activate Stat5 through the IL-2R pathway in an autocrine manner induced by elevated IL-2 production (40). Thus, our results provide a potential mechanism for $p12^I$-mediated Stat5 activation.

$p12^I$ is probably not involved in the transformation process of HTLV-1-infected T cells. mRNA of ORF I can be detected in both IL-2-dependent and -independent HTLV-1-infected T-cell lines (4–7, 17, 25), and $p12^I$ is not necessary for immortalization of HTLV-1-infected cells in vitro (11, 45). Therefore, the elevated IL-2 secretion mediated by expression of $p12^I$ may promote HTLV-1-infected T cells to proliferate through an autocrine or paracrine mechanism and allow HTLV-1 infection to subsequently spread more effectively.

Using a variety of $p12^I$ truncation mutants, we identified two positive regions (aa 33 to 47 and 87 to 99) and two negative regions (aa 1 to 14 and 70 to 86) in the viral protein for NFAT activation. An SH3-binding domain (PXXP) is contained in each individual region. Further analysis with point mutants that replaced prolines with alanines in each PXXP motif demonstrated that the third PXXP motif ($P_{70}XXP_{73}$) is responsible for the inhibitory effect of region aa 70 to 86 on NFAT activation. Interestingly, we have recently identified a conserved PxIxIT calcineurin-binding motif, encompassing the third PXXP motif, in $p12^I$ that binds to calcineurin (22). Wild-type $p12^I$ and deleted mutants, which contained the motif, bound calcineurin. In addition, an alanine substitution mutant ($p12^I$ AxAxAA) had greatly reduced binding affinity for calcineurin.
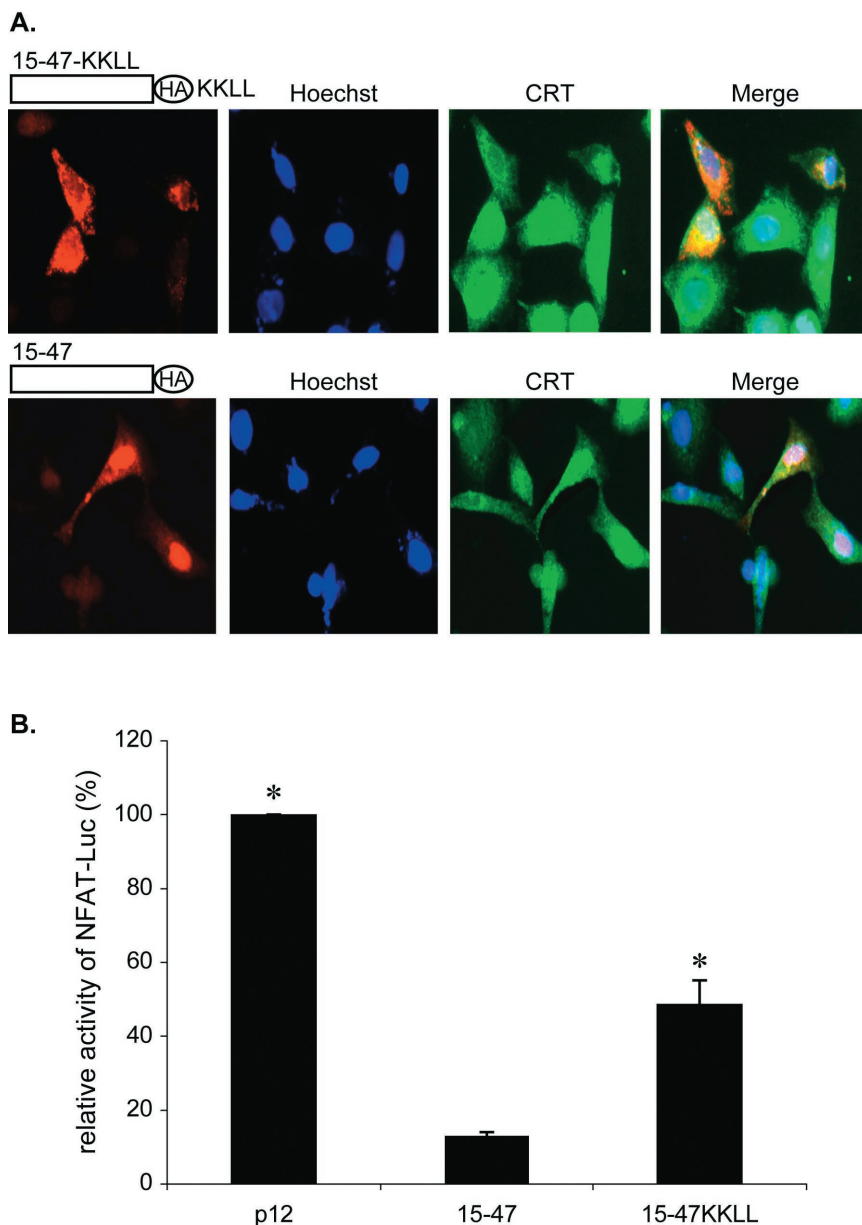
FIG. 10. ER targeting partially restores the NFAT activation mediated by mutant p12^I 15-47. (A) Localization of mutant 15-47 and chimeric protein 15-47KKLL. An ER targeting signal, KKLL, redirected the chimeric protein 15-47KKLL to ER compartments stained with the ER marker, calreticulin (CRT). The nuclei were stained with Hoechst stain. (B) Relative NFAT activities mediated by p12^I, mutant 15-47, and chimeric protein 15-47KKLL. Data are presented as relative NFAT luciferase activities compared to wild-type p12^I. Values are means of three independent transfection experiments. Statistical significance was analyzed by using the Student t test. *, $P < 0.05$.

Nef also contains the SH3-binding domain, and this motif is required for the binding between Nef and multiple cellular proteins, including Hck and PAK, and is necessary for Nef-mediated NFAT activation (27, 29, 30). Thus, further studies designed to identify possible binding partners of p12^I and the role of these motifs in HTLV-1 infection will be necessary to clarify the role of specific motifs in T-cell activation. Finally, we observed partially restored NFAT activation by tagging the mutant 15-47 with an ER targeting signal, KKLL. This finding demonstrates the requirement of ER localization in p12^I mediated NFAT activation, further indicating the role of this protein in modulating the ER calcium homeostasis.

In summary, expression of p12^I in primary T lymphocytes and Jurkat T cells promotes IL-2 production during T-cell activation, suggesting the important role of this viral accessory protein in early HTLV-1 infection in vivo.

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