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Negative Regulation of the NFAT1 Factor by CD45: Implication in HIV-1 Long Terminal Repeat Activation¹

Benoit Barbeau,² Gilles A. Robichaud,² Jean-François Fortin,² and Michel J. Tremblay³

HIV-1 gene regulation is greatly dependent on the presence of the -104/-81 enhancer region which is regulated by both NF- κ B and NFAT transcription factors. We have found that a greater induction in HIV-1 long terminal repeat-driven gene expression was observed upon PMA/ionomycin (Iono) stimulation of a CD45-deficient cell line (J45.01) in comparison to the parental Jurkat cells. Unlike NF- κ B which was not affected by the absence of CD45, NFAT showed a much greater augmentation in nuclear translocation and transcriptional activity in J45.01 cells upon PMA/Iono stimulation. PMA/Iono-induced NFAT activation, NFAT translocation and calcium influx peaked at similar time points for both Jurkat and J45.01 cell lines. The NFAT-dependent promoters from the *IL-2* and *TNF- α* genes were also more potently activated by PMA/Iono in J45.01 cells. Interestingly, higher levels of intracellular calcium were consistently demonstrated in PMA/Iono-induced CD45-deficient cell lines (J45.01 and HPB45.0). Furthermore, PMA/Iono induction of calcium mobilization in both Jurkat and J45.01 cell lines was observed to be EGTA-sensitive. Mechanistic studies revealed that CD3 ζ and ZAP-70 were more heavily tyrosine phosphorylated in J45.01 cells than Jurkat cells. Analysis of the HIV-1 enhancer by EMSAs demonstrated that the bound NFAT complex was present at higher levels in J45.01 nuclear extracts and that the NFAT1 member was predominant. In conclusion, our results indicate that NFAT activation by stimuli acting in a more distal fashion from the TCR-mediated signaling pathway can be down-regulated by CD45 and that this CD45-dependent regulation in turn affects HIV-1 long terminal repeat activation. *The Journal of Immunology*, 2001, 167: 2700–2713.

Human immunodeficiency virus type 1 replication is intimately linked to the activity of its promoter region positioned in the 5' long terminal repeat (LTR)⁴ sequence. Through the use of this LTR region, HIV-1 interacts with cellular transcriptional factors available in the nucleus which in turn, dictate viral transcription. In the LTR, a crucial enhancer region positioned at -104/-81 has been shown to be required for viral replication in both T cells and monocytes following cellular activation (1–4). The enhancer harbors two tandem conserved motifs which permit the recruitment of the well-known NF- κ B complex (5).

The NF- κ B (Rel) family is composed of several members which all share a 300-aa domain, termed the Rel homology domain, containing the DNA-binding and dimerization domains and the nuclear localization signal (NLS) (6, 7). These members bind to a

DNA consensus sequence as either homo- or heterodimers, the most important complex for HIV-1 LTR activity being the p50/p65 heterodimer (8). Activation of NF- κ B occurs upon its dissociation from the cytoplasmic I κ B α repressor, this latter being targeted for degradation after phosphorylation of serine residues 32 and 36 and subsequent ubiquitination (9–14). The NLS of the nonassociated NF- κ B complex is then exposed, which in turn leads to free translocation of the complex from the cytoplasm to the nucleus (15–17). Although NF- κ B is a key regulator in HIV-1 expression, the NFAT has more recently received a great deal of attention in its role in HIV-1 regulation and expression (18–21).

NFAT is another family of Rel-related transcription factors which are turned on during early events of T cell activation. The NFAT family is composed of five members: NFAT1, NFAT2, NFAT3, NFAT4, and the recently isolated NFAT5 (22). Members NFAT1 through NFAT4 can themselves, through alternative splicing, produce different isoforms (23–25). Several of these NFAT family members are expressed in both normal human T cells (26) and the classical Jurkat T cell line (23, 27–29). The hallmark of NFAT activation is its inducibility by agents raising intracellular Ca²⁺ content (26, 30, 31). A sustained increase in intracellular Ca²⁺ activates the serine/threonine phosphatase calcineurin, which thereby dephosphorylates NFAT, exposing its NLS sequence and consequently permitting nuclear import as well as increasing its DNA-binding affinity (32, 33). The role of calcineurin in NFAT activation has been further determined by the demonstration that two known immunosuppressors, FK506 and cyclosporin A, could block NFAT activation through the inhibition of calcineurin activity (32, 34–36). Many studies have also shown that NFAT activity is mediated through the formation of a multi-proteic complex composed of a cytoplasmic unit (NFAT) and the nuclear AP-1 (37). The NFAT-interacting ubiquitinary AP-1 transcriptional factor itself is a dimer resulting from the association of nuclear proteins of the *fos* and *jun* families (27, 37). Many studies have been conducted showing that this latter factor is necessary for

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⁴ Abbreviations used in this paper: LTR, long terminal repeat; NLS, nuclear localization signal; PTK, protein tyrosine kinase; PTP, protein tyrosine phosphatase; Iono, ionomycin; PLC γ 1, phospholipase C γ 1; PKC, protein kinase C; RIPA, radioimmuno-precipitation assay; PVDF, polyvinylidene difluoride.

a full and adequate NFAT response (38–40), although certain promoters have indicated the existence of functional NFAT binding sites with no requirement for a nearby AP-1 binding site (41).

Although the implication of NFAT in HIV-1 LTR regulation has led to early contradictory results (42–45), Kinoshita et al. (46) have demonstrated that the NFAT2 family member was indeed an important regulatory factor of the HIV-1 enhancer region and that it further cooperated with NF- κ B (46). The involvement of NFAT in the positive regulation of the HIV-1 LTR has furthermore been suggested in primary human mononuclear cells through the use of phosphodiesterase type IV inhibitors and different NFAT inhibitors (19, 21). However, recently, the NFAT1 member has been hypothesized to negatively modulate HIV-1 LTR activity, thus questioning the function and role of the different NFAT members in HIV-1 LTR activity (47).

For many years now, studies have narrowed down signaling pathways in activated T cells leading to the activation of transcriptional factors positively regulating HIV-1 LTR activity. One of the early events following TCR-mediated T cell activation is the subsequent increase in tyrosine phosphorylation of specific proteins (48–50). Intracellular phosphotyrosine content is generally regulated by a balance between protein tyrosine kinase (PTK) and protein tyrosine phosphatase (PTP) enzymatic activities. CD45 is a transmembrane PTP and represents >10% of the total cell surface protein content in lymphocytes (51). CD45 is also well known to participate in the early events initiating T cell signaling, playing a pivotal role in T cell antigenic response (52), T cell proliferation and differentiation (53), PTK signaling, intracellular calcium (Ca^{2+}) mobilization, and TCR-CD28 signaling cascades (54, 55). CD45 is expressed in at least eight different isoforms generated through alternative RNA splicing (54). Being biochemically associated to the TCR-CD28 complex, CD45 has several substrates which interact with the cell surface TCR complex (56, 57). Two of the major substrates for CD45 are the PTK p56^{lck} and p59^{lyn}. Although it was widely accepted that CD45 induced PTK activation by dephosphorylating a negative regulatory site (57–61), more data have suggested that CD45 might be acting negatively on overall p56^{lck} activity through the dephosphorylation of its autophosphorylation site (i.e., Tyr³⁹⁴) (62–64). Regulation of the TCR-induced signaling pathway by CD45 has also been demonstrated through its ability to dephosphorylate the CD3 ζ chain of the TCR complex (56, 65).

One study has focused on the possible role played by CD45 on overall HIV-1 LTR activity (66). In this analysis, Baur et al. (66) indicated that basal HIV-1 LTR-mediated gene transcription was more elevated in CD45-deficient T cell lines because of higher constitutive levels of the translocated NF- κ B complex in these cell lines in comparison to their parental CD45-positive counterpart. However, no implications of CD45 in the regulation of the HIV-1 LTR following stimulation was assessed in this latter study. Since the CD45 PTP is highly implicated in TCR signaling events, we therefore set out to investigate the possible role of the CD45 molecule in the modulation of transcriptional factors regulating HIV-1 gene transcription following T cell activation. In this study, we demonstrate that CD45 suppresses NFAT activation following treatment with the phorbol ester PMA and the ionomycin (Iono) Ca^{2+} ionophore. Intracellular Ca^{2+} mobilization and AP-1 activation induced by PMA/Iono were concomitantly reduced in CD45-positive cells. Tyrosine phosphorylation of both CD3 ζ and ZAP-70 was more pronounced in the CD45-negative cell line. Furthermore, our results suggest that CD45 negatively regulates HIV-1 LTR activity via a down-modulation of the NFAT1 member binding preferentially to the HIV-1 enhancer region. These findings bring important insights on TCR signaling events and

most of all upon the regulation of the HIV-1 LTR following T cell signaling and activation.

Materials and Methods

Cell lines

The lymphoid T cell lines used include Jurkat (clone E6-1) (67), J45.01, HPB.ALL, and HPB45.0. J45.01 is a derivative of the Jurkat leukemic T cell line that is deficient in CD45 expression (68). HPB45.0 is a natural variant of HPB.ALL (69) selected for the absence of CD45 expression (70). These cell lines were provided by Dr. A. Weiss (Howard Hughes Medical Center, San Francisco, CA). We also made use of H45.01 cells, another CD45-deficient variant of the HPB.ALL parental cell line (provided Dr. W. J. Esselman, Michigan State University, East Lansing, MI) (71). Cell lines were maintained in complete culture medium made of RPMI 1640 supplemented with 10% FBS (HyClone Laboratories, Logan, UT), glutamine (2 mM), penicillin G (100 U/ml), and streptomycin (100 $\mu\text{g}/\text{ml}$).

Plasmids and Abs

The plasmid pLTR-LUC was kindly provided by Dr. K. L. Calame (Columbia University, NY) and contains the luciferase reporter gene under the control of the complete HXB2-derived HIV-1 LTR (72). pNFAT-LUC containing the minimal *IL-2* promoter with three (3) tandem copies of the NFAT-binding site as well as the full-length *IL-2* promoter pIL-2 plasmid (31) were kind gifts from Dr. G. Crabtree (Howard Hughes Medical Institute, Stanford, CA). pNF- κ B-LUC (purchased from Stratagene, La Jolla, CA) contains five consensus NF- κ B-binding sequences placed upstream to the luciferase gene along with a minimal promoter. The pTNF- α -LUC vector was kindly provided by Dr. A. Rao (Harvard Medical School, Boston, MA) (41). The CD45-expressing vector (pSP.SR α .LCA1) and the backbone vector (pSP.SR α .2) were kindly sent by Dr. H. Saito (Division of Tumor Immunology, Dana-Farber Institute, Boston MA) (73). Rabbit antisera raised against peptides from NFAT1 and NFAT4 (26) or against the p50 and p65 subunits of NF- κ B were obtained from Dr. N. Rice (National Cancer Institute, Frederick, MD). Polyclonal anti-NFATc (NFAT2) Abs were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Hybridomas producing anti-CD3 OKT3 (specific for the θ -chain of the CD3 complex) and anti-CD45 GAP8.3 Abs were obtained from the American Type Culture Collection (Manassas, VA). Abs from these hybridomas were purified with mAbTrap protein G affinity columns according to the manufacturer's instructions (Pharmacia LKB Biotech, Uppsala, Sweden). Purified anti-CD28 Abs (clone 9.3) were a generous gift from Dr. J. A. Ledbetter (Bristol-Myers Squibb Pharmaceutical Research Institute, Princeton, NJ) (74). Purified goat anti-mouse IgG Abs were purchased from Jackson ImmunoResearch (West Grove, PA). Anti-phosphotyrosine Abs (clone 4G10) were obtained from Upstate Biotechnology (Lake Placid, NY). Anti-CD3 ζ Abs (clone 6B10.2) were purchased from Santa Cruz Biotechnology. Anti-ZAP-70 and anti-lck Abs were generously provided by Dr. A. Y. Tsygankov (Temple University, Philadelphia, PA).

Transfections and reporter gene assays

Transient transfections were performed using the DEAE-dextran method as previously described (75). To minimize variations in plasmid transfection efficiencies, cells were transfected in bulk and were next separated into various treatment groups at a density of 10^5 cells/well (100 μl) in 96-well flat-bottom plates. Cells were either left unstimulated or treated with PHA (PHA-P at 3 $\mu\text{g}/\text{ml}$; Sigma, St. Louis, MO), PMA (20 ng/ml; Sigma), Iono (at 1 μM ; Calbiochem, La Jolla, CA), anti-CD3 Ab (clone OKT3 at 3 $\mu\text{g}/\text{ml}$), and anti-CD28 Ab (clone 9.3 at 1 $\mu\text{g}/\text{ml}$) along with a goat anti-mouse IgG (5 $\mu\text{g}/\text{ml}$) in a final volume of 200 μl . Next, cells were incubated at 37°C for 8 h unless otherwise specified. Luciferase activity was determined following a previously described protocol (75). Fold induction was obtained by calculating the ratio between measured relative light units of treated over untreated samples.

Preparation of nuclear extracts and EMSA

Cells were either left untreated or incubated for the indicated time at 37°C with the combination of PMA (20 ng/ml)/Iono (1 μM). Incubation with the various stimulating agents was terminated by the addition of ice-cold PBS, and nuclear extracts were prepared according to the previously described microscale preparation protocol (76). Protein concentrations were determined by the bicinchoninic assay with a commercial protein assay reagent (Pierce, Rockford, IL). Nuclear extracts (10 μg) were incubated for 20 min at room temperature in 20 μl of 1 \times binding buffer (100 mM HEPES (pH 7.9), 40% glycerol, 10% Ficoll, 250 mM KCl, 10 mM DTT, 5 mM EDTA, 250 mM NaCl, 2 μg of poly(dI-dC), and 10 μg of nuclease-free BSA

fractionV) containing 0.8 ng of γ - 32 P-labeled dsDNA oligonucleotide. The following dsDNA oligonucleotides were used as probes and/or competitors: the distal NFAT binding site from the murine IL-2 promoter (5'-TCGAGCCCAAAGGAAAATTTGTTTCATG-3'); the consensus binding site for AP-1 (5'-CGCTTGATGACTCAGCCGAA-3'); the consensus NF- κ B binding site (5'-ATGTGAGGGGACTTCCAGGC-3'); the enhancer region (-107/-77) from the NL4-3 strain HIV-1 LTR (5'-CAAGGGACTTCCGCTGGGACTTCCAGGG-3'); and the consensus binding site for C/EBP (5'-TCGAGATGCGCAATCTGCA-3'). Oligonucleotides were either purchased (C/EBP; Santa Cruz Biotechnology) or synthesized in-house. DNA-protein complexes were resolved from free labeled DNA by electrophoresis in native 4% (w/v) polyacrylamide gels. The gels were subsequently dried and autoradiographed. Cold competition assays were conducted by adding a 100-fold molar excess of unlabeled dsDNA oligonucleotide simultaneously with the labeled probe. Supershift assays were performed by preincubation of nuclear extracts with 1 μ l of Ab in the presence of all of the components of the binding reaction for 30 min on ice before the addition of the labeled probe.

Immunoprecipitations and Western blot analysis

Cells (5×10^6) were treated (as described in the figure legends) and lysed using 0.5 ml of radioimmunoprecipitation assay (RIPA) lysis buffer ($1 \times$ PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS along with freshly added 10 μ l/ml PMSF (10 mg/ml), 30 μ l/ml aprotinin, and 10 μ l/ml sodium orthovanadate (100 mM)). Cells extracts were then incubated on ice for 20 min and precleared of cellular debris by centrifugation at $10,000 \times g$. From the supernatant of these cellular extracts, 500 μ g of protein extract was then combined with 1-ml total volume of RIPA buffer where 1 μ g of primary Ab was added and incubated at 4°C with continuous mixing for 1 h. Protein A/G-agarose (20 μ l) was added to the extracts and mixed at 4°C for 1 h. Immunoprecipitates were then washed four times with 1 ml of RIPA buffer by centrifugation at $1,000 \times g$ and then resuspended in 40 μ l of 1 \times electrophoresis sample SDS-PAGE buffer. Samples were heated at 95°C and migrated on a 12% SDS-polyacrylamide gel. Proteins were then transferred to a polyvinylidene difluoride (PVDF) membrane. The membrane was then blocked with 3% gelatin and incubated with primary Abs followed by conjugated secondary Abs. Signals were revealed with an ECL kit according to the manufacturer's protocol (Amersham, Arlington Heights, IL).

Flow cytometric analysis of intracellular Ca^{2+} and CD45 cell surface expression

Cells (1×10^7) were washed once and resuspended in RPMI 1640 supplemented with 10% FBS at a concentration of 1×10^7 cells/ml. The cell permeant calcium indicator Indo-1AM (Molecular Probes, Eugene, OR) was added to the cells at a concentration of 3 μ M and the cells were incubated in the dark at room temperature for 1 h with moderate shaking. Cells were then washed twice with ice-cold serum-free/phenol red-free Opti-Mem medium (Life Technologies, Rockville, MD) and resuspended in Opti-Mem at a concentration of 1×10^6 cells/ml. Thereafter, prewarmed cells (1×10^6) were stimulated with PHA (3 μ g/ml), OKT3 (3 μ g/ml)/9.3 (1 μ g/ml)/goat anti-mouse IgG (5 μ g/ml), PMA (20 ng/ml)/Iono (1 μ M), or thapsigargin (1 μ M; Sigma) and calcium content was then analyzed with an EPICS ELITE ESP apparatus (Beckman-Coulter, Miami, FL). In some experiments, EGTA (8 mM) treatment was performed after 5 min of cell activation. The violet:blue ratios, representing the Ca^{2+} -bound to Ca^{2+} -unbound Indo-1 signals, were then continuously monitored over a 10-min period and analyzed using the 1.5 version of the System 2 software (Beckman-Coulter). Data are represented as the geometric mean of the violet:blue ratio over time, using the WinMDI v2.8 freeware (J. Trotter, The Scripps Institute, La Jolla, CA). Detection of CD45 cell surface expression was performed by FACS analysis as previously described (75) with the anti-CD45 GAP8.3 monoclonal Ab.

Results

HIV-1 LTR activity is very tightly regulated by tyrosine phosphorylation events as we and others have previously demonstrated (75, 77). Furthermore, it has been suggested that CD45, which accounts for most of the T cell surface-associated PTP activity, was negatively affecting the basal level of HIV-1 LTR activity (66). In this study, we were interested in looking at the effect of CD45 on HIV-1 LTR activation by agents which induced T cell stimulation in either a TCR-dependent or -independent fashion.

HIV-1 LTR activity is more strongly induced by PMA/Iono in CD45-negative cells

A plasmid containing the HIV-1 LTR positioned 5' to the luciferase reporter gene was initially transfected in the CD45-positive Jurkat cell line and its CD45-negative derivative, the J45.01 cell line. Luciferase activity was subsequently measured after activation of the transfected cells with different stimuli. As shown in Fig. 1, PHA alone led to an increase in luciferase activity in Jurkat cells (3-fold) which was not detectable in J45.01 cells. This was expected given the CD45-dependent nature of T cell stimulation occurring through TCR-dependent activators (54). Similar results were obtained with the combined addition of anti-CD3 and anti-CD28 Abs. However, surprisingly, stimulation of HIV-1 LTR activity with a combination of the PMA phorbol ester and the calcium ionophore Iono was found to be more pronounced in CD45-deficient cells than in Jurkat cells (31.6- vs 14.7-fold, respectively). These results hence suggested that CD45-negative cells were more responsive to HIV-1 LTR activation by a PMA/Iono stimulation.

NF- κ B activation by PMA/Iono is not affected by CD45

We next wanted to investigate the mechanism which entailed this stronger HIV-1 LTR induction in CD45-negative cells. Since PMA/Iono is known to lead to the activation of NF- κ B, Jurkat and J45.01 cells were then transfected with a plasmid harboring five tandem consensus NF- κ B binding sites positioned upstream from a minimal promoter followed by the luciferase reporter gene (pNF- κ B-LUC). As demonstrated in Fig. 2A, NF- κ B-dependent luciferase activity was induced in Jurkat cells by all stimulating agents, being more intense following a PMA/Iono stimulation. However, when J45.01 cells were similarly treated, a marked diminution in luciferase activity induction was apparent in PHA-treated cells (0.7-fold). PMA treatment was similarly active in both cell lines while the PHA/PMA induction led to a reduced induction in J45.01 cells which was then comparable to the level of induction by PMA alone for both cell lines. When PMA/Iono treatment was compared, no differences in terms of luciferase induction between Jurkat and J45.01 cells were measured (1149- vs 1166-fold for Jurkat- and J45.01-transfected cells, respectively). To substantiate this latter observation, EMSA were performed with a NF- κ B-labeled probe. As presented in Fig. 2B, no major differences in band intensity specific to the NF- κ B probe were observed between PMA/Iono-stimulated Jurkat (lane 3) and J45.01 cells (lane 5). The NF- κ B complex was

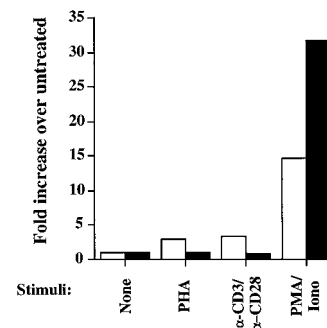


FIGURE 1. HIV-1 LTR activation is greatly induced by PMA/Iono in J45.01 cells. Jurkat (□) and J45.01 (■) cells were transfected with pLTR-LUC and either left untreated or treated with PHA (3 μ g/ml), anti-CD3 (3 μ g/ml)/anti-CD28 (1 μ g/ml) in the presence of a goat anti-mouse IgG (5 μ g/ml), or PMA (20 ng/ml)/Iono (1 μ M). After 8 h of stimulation, cells were assessed for luciferase activity. Results are presented as fold induction of luciferase activity over untreated samples from the calculated means of three different lysed cell samples in the same experimental setting. These results are representative of three different experiments.

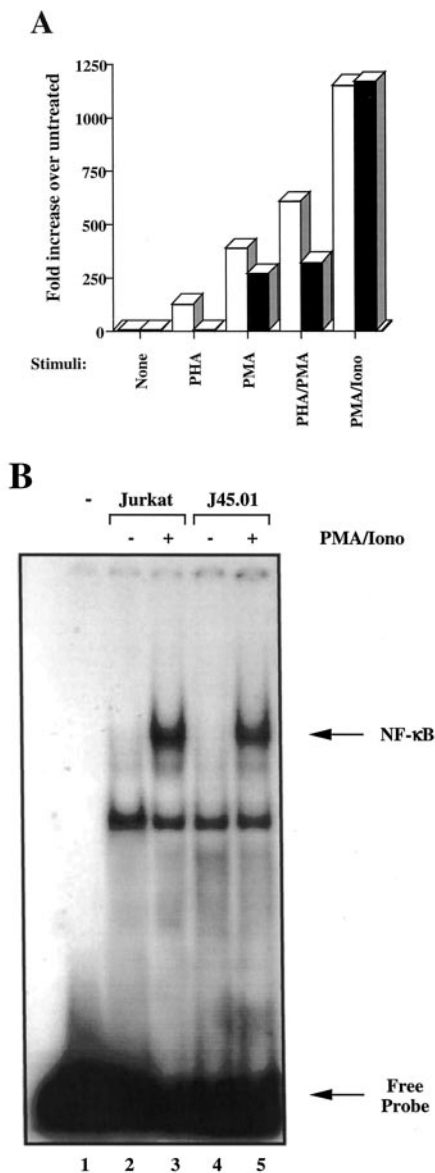


FIGURE 2. CD45 does not modulate PMA/Iono-induced NF- κ B activation. *A*, Jurkat (□) and J45.01 (■) cells were transfected with pNF- κ B-LUC and were either left untreated or treated with PHA (3 μ g/ml), PMA (20 ng/ml), PHA (3 μ g/ml)/PMA (20 ng/ml), or PMA (20 ng/ml)/Iono (1 μ M). After 8 h of stimulation, cells were assessed for luciferase activity. Results are presented as fold induction of luciferase activity over untreated samples from the calculated means of three different lysed cell samples in the same experimental setting. These results are representative of three different experiments. *B*, Jurkat and J45.01 cells were left untreated or stimulated with PMA (20 ng/ml)/Iono (1 μ M) for 1 h. Nuclear extracts from Jurkat (lanes 2 and 3) and J45.01 (lanes 4 and 5) which were either untreated (lanes 2 and 4) or PMA/Iono-treated (lanes 3 and 5) were incubated with a NF- κ B-labeled probe to be finally analyzed on a 4% native polyacrylamide gel. Probe only was run in lane 1. Arrows on the right indicate the NF- κ B-specific complex and the free probe.

specifically outcompeted by an excess of cold NF- κ B oligonucleotide (data not shown). These data hence suggested that NF- κ B was not the predominant transcription factor responsible for the higher induction of HIV-1 LTR activity observed in PMA/Iono-treated J45.01 cells.

NFAT-dependent transcription is more potently activated in CD45-deficient J45.01 cells after PMA/Iono treatment

The NFAT transcription factor has been reported to synergize with NF- κ B in up-regulating HIV-1 LTR activity (46). To assess its role

in the observed difference in PMA/Iono-mediated HIV-1 LTR stimulation, we first transfected both Jurkat and J45.01 cell lines with the pNFAT-LUC plasmid. This vector contains three tandem repeats of the human *IL-2* promoter distal NFAT binding site in the context of the minimal *IL-2* promoter itself, all of which are positioned upstream of the luciferase reporter gene. After stimulation of the transfected cells, NFAT activation by both PHA and PHA/PMA was absent in CD45-negative cells as opposed to the Jurkat parental cell line, which displayed an increase in luciferase activity (Fig. 3*A*). As previously described (28, 78), PMA on its own was not sufficient to lead to any significant induction in NFAT-dependent luciferase activity. However, remarkably, the induction of luciferase activity was much more pronounced in J45.01 cells in comparison to Jurkat cells when these cells were treated with PMA/Iono (from a 171- to a 666-fold increase in Jurkat and J45.01 cells, respectively). To further corroborate the implication of CD45 in this down-modulation of NFAT activation, J45.01 cells were cotransfected with a CD45-expressing vector (pSP.SR α .LCA1) along with the pNFAT-LUC vector. As shown in Fig. 3*B*, cotransfection of the CD45 expression vector diminished the induction of NFAT-driven luciferase activity in comparison to cells cotransfected with the

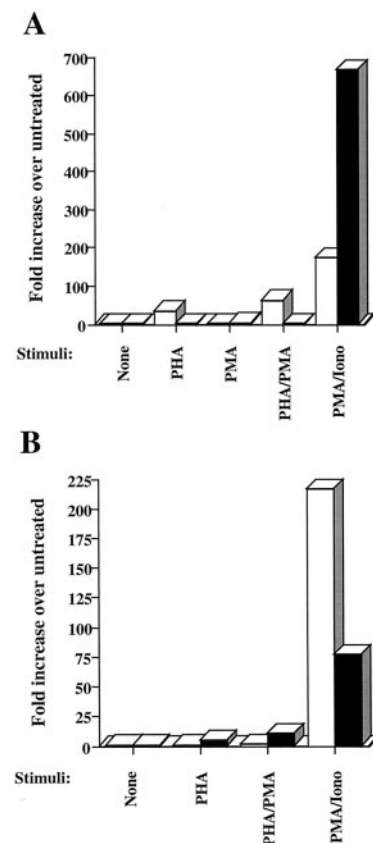


FIGURE 3. CD45 negatively regulates PMA/Iono-induced NFAT activation. *A*, Jurkat (□) and J45.01 (■) cells were transfected with pNFAT-LUC and were either left untreated or treated with PHA (3 μ g/ml), PMA (20 ng/ml), PHA (3 μ g/ml)/PMA (20 ng/ml), or PMA (20 ng/ml)/Iono (1 μ M). *B*, J45.01 cells were cotransfected with pNFAT-LUC and pSP.SR α .2 (□) or pSP.SR α .LCA1 (■) and were either left untreated or treated with PHA (3 μ g/ml), PHA (3 μ g/ml)/PMA (20 ng/ml), or PMA (20 ng/ml)/Iono (1 μ M). After 8 h of stimulation, cells were assessed for luciferase activity. Results are presented as fold induction of luciferase activity over untreated samples from the calculated means of three different lysed cell samples in the same experimental setting. Results in *A* and *B* are representative of three different experiments.

pSP.SR α .2 empty vector (from 217-fold down to 77-fold). Successful cell surface expression of CD45 was confirmed by the fact that NFAT activation by both PHA and PHA/PMA was restored after transfection of the CD45-expressing vector (attaining 5.1- and 10.3-fold activation, respectively). In addition, the presence of CD45 on the cell surface of these transfected cells was detected for a certain percentage of the cells by FACS analysis (data not shown). Our results hence indicated that NFAT induction by PMA/Iono was negatively regulated by CD45 and suggestively could affect HIV-1 LTR activity.

NFAT translocation and AP-1 activation are more pronounced in PMA/Iono-treated CD45-negative cell lines

We next sought to further confirm the observation that NFAT might be more intensively induced by PMA/Iono in CD45-negative cell lines by performing EMSA experiments. Two different CD45-negative cell lines were tested in these assays, i.e., the J45.01 and the HPB45.0 cell lines along with their respective parental cell lines (Jurkat and HPB.ALL, respectively). Incubation of a NFAT-specific labeled probe with nuclear extracts from PMA/Iono-stimulated cells led to the appearance of a unique band corresponding to the NFAT factor (Fig. 4A, compare lanes 3, 5, 10, and 12 with lanes 2, 4, 9, and 11, respectively). Interestingly, ex-

tracts from both J45.01- and HPB45.0 CD45-negative cells had a more intense signal than their respective CD45-positive counterparts (Fig. 4A, compare lanes 5 and 12 with lanes 3 and 10, respectively). The NFAT-binding complex present in the J45.01 extracts was found to be specifically outcompeted by increasing concentrations of cold excess of NFAT oligonucleotide (Fig. 4A, lanes 6 and 7) while it was unaffected by a 100-fold excess of an oligonucleotide corresponding to the C/EBP binding site (lane 8). These results hence agreed with the results obtained in transfection experiments as for the potential of CD45 to regulate NFAT-driven gene expression. Since the transcription factor AP-1 has been known to be an important cooperating factor for NFAT (40, 79), EMSA experiments were performed with a probe consisting of an AP-1-binding site (Fig. 4B). Again, the AP-1 complex was found to be more abundant in nuclear extracts from PMA/Iono-induced J45.01 cells than from stimulated Jurkat cells (Fig. 4B, compare lane 3 with lane 5). This signal was outcompeted by the addition of 100-fold excess of cold AP-1 oligonucleotide (Fig. 4B, lane 6). These results hence clearly showed that CD45-negative cells treated with PMA/Iono demonstrated a higher level of NFAT translocation in two different cell settings and that a similar greater increase in AP-1 activation was concomitantly found in the tested J45.01 cells.

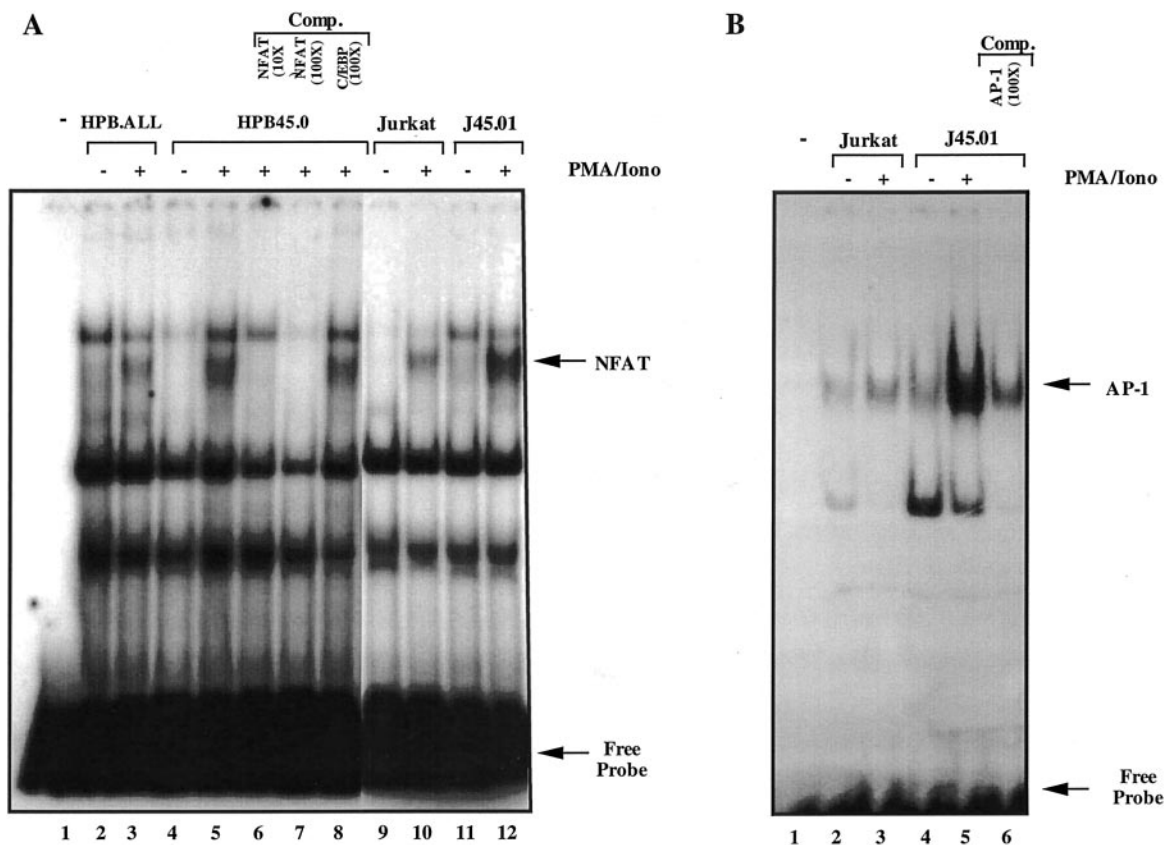


FIGURE 4. Higher levels of nuclear NFAT and AP-1 in CD45-deficient cell lines after PMA/Iono stimulation. Cells were either left untreated or treated with PMA (20 ng/ml)/Iono (1 μ M) for 1 h. **A**, Nuclear extracts from HPB.ALL (lanes 2 and 3), HPB45.0 (lanes 4–8), Jurkat (lanes 9 and 10), and J45.01 (lanes 11 and 12) cells were incubated with a NFAT-labeled probe. Competition experiments were conducted with either 10- or 100-fold excess of cold NFAT oligonucleotide (lanes 6 and 7, respectively) or 100-fold excess of cold C/EBP oligonucleotide (lane 8). Samples were run on a 4% native polyacrylamide gel. PMA/Iono treatment is indicated above each lane (–, untreated; +, PMA/Iono treated). Lane 1 contains the probe only. Arrows at the right indicate the position of the specific NFAT complex and the free probe. **B**, Nuclear extracts from Jurkat (lanes 2 and 3) and J45.01 (lanes 4–6) cells were incubated with an AP-1-labeled probe. Competition experiments were conducted with 100-fold excess of cold AP-1 oligonucleotide (lane 6). Samples were run on a 4% native polyacrylamide gel. PMA/Iono treatment is indicated above each lane (–, untreated; +, PMA/Iono treated). Lane 1 contains the probe only. Arrows at the right indicate the position of the specific AP-1 complex and the free probe.

Greater activation of the promoter regions of IL-2 and TNF- α in J45.01 cells following PMA/Iono stimulation

To better demonstrate that NFAT was indeed more active in CD45-negative cells upon PMA/Iono stimulation, we tested promoter regions known to be importantly regulated by NFAT (Fig. 5). The vector pIL-2-LUC containing the full-length *IL-2* promoter region was first transfected in Jurkat and J45.01 cells and subsequently activated. As depicted in Fig. 5A, both PHA and PHA/PMA stimulations of *IL-2* promoter-driven gene expression were importantly hampered in transfected J45.01 cells. However, as expected, PMA/Iono activation of the transfected cells resulted in higher fold inductions in the CD45-negative cells when compared with the CD45-positive cells. Since the interaction between NFAT and AP-1 is important for the action of NFAT in this promoter context, we then decided to determine whether a promoter containing a functional NFAT binding site independent of AP-1 interaction was more potently activated by PMA/Iono in CD45-negative cells. The *TNF- α* promoter has been previously reported to be regulated by NFAT in an AP-1-independent fashion (41). A pTNF- α -LUC vector was hence transfected in both Jurkat and J45.01 cells (Fig. 5B). Upon stimulation, the activation of this promoter by PHA or PHA/PMA was again greatly perturbed by the absence of CD45. On the other hand, the activation of the *TNF- α* promoter by PMA/Iono was higher in J45.01 cells than in Jurkat cells. Our results thus demonstrated that NFAT-regulated promoters are also more responsive to a PMA/Iono treatment in the absence of CD45. In addition, these data indicated that an increase in AP-1 activation, as demonstrated above,

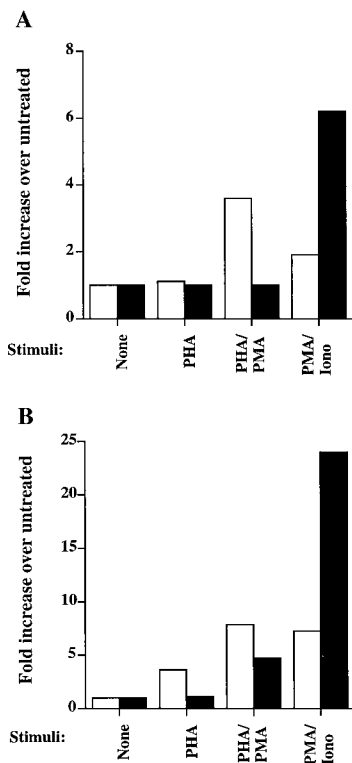


FIGURE 5. Both *IL-2* and *TNF- α* promoters are more potently induced in J45.01 cells by PMA/Iono. Jurkat (□) and J45.01 (■) cells were transfected with pIL-2-LUC (A) or pTNF- α -LUC (B) and were either left untreated or treated with PHA (3 μ g/ml), PHA (3 μ g/ml)/PMA (20 ng/ml), or PMA (20 ng/ml)/Iono (1 μ M). After 8 h of stimulation, cells were assessed for luciferase activity. Results are presented as fold induction of luciferase activity over untreated samples from the calculated means of three different lysed cell samples in the same experimental setting. Results in A and B are representative of two different experiments.

cannot solely account for the more potent activation of *IL-2* and *TNF- α* promoters in the CD45-deficient cell line.

Kinetic analyses of NFAT-dependent gene transcription and nuclear translocation after PMA/Iono treatment

We then wanted to determine whether the increase in both NFAT-driven luciferase activity and NFAT translocation specific to CD45-negative cells was also paralleled by a different type of kinetics of activation induced by PMA/Iono. Time kinetic experiments were thus performed for both Jurkat and J45.01 cells transfected with the pNFAT-LUC vector. As depicted in Fig. 6A (left panel), in terms of luciferase activity, time kinetics were observed to be similar for both cell lines after PMA/Iono stimulation. However, higher luciferase activity induction was constantly observed in J45.01 cells following PMA/Iono treatment. In comparison, a typical stimulation by the anti-CD3/anti-CD28 combination demonstrated the requirement of CD45 for this type of stimulation

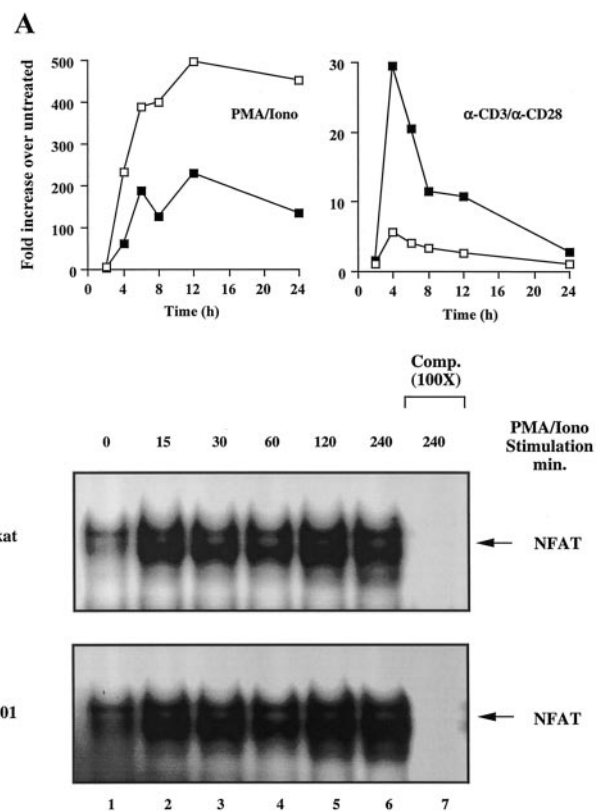


FIGURE 6. No differences in time kinetic responses between Jurkat and J45.01 cells for both NFAT translocation and NFAT-driven luciferase activity. A, Jurkat (■) and J45.01 (□) cells were transfected with pNFAT-LUC and were either left untreated or treated with PMA (20 ng/ml)/Iono (1 μ M) (left panel) or with anti-CD3 (3 μ g/ml)/anti-CD28 (1 μ g/ml) in the presence of a goat anti-mouse IgG (5 μ g/ml) (right panel). Luciferase activity was assessed after 2, 4, 6, 8, 12, or 24 h of treatment. Results are presented as fold induction in luciferase activity over untreated samples from the calculated means of three different lysed cell samples in the same experimental setting. These results are representative of two different experiments. B, Jurkat and J45.01 cells were either left untreated or stimulated with PMA (20 ng/ml)/Iono (1 μ M) for 0 (lanes 1), 15 (lanes 2), 30 (lanes 3), 60 (lanes 4), 120 (lanes 5), or 240 min (lanes 6). Nuclear extracts from Jurkat (upper panel) or J45.01 (lower panel) cells were then incubated with a NFAT-labeled probe to be finally analyzed on a 4% native polyacrylamide gel. Competition experiments with cold excess of the NFAT oligonucleotide were performed with nuclear extracts from Jurkat and J45.01 cells stimulated with PMA/Iono for 240 min (lanes 7). Arrows on the right indicate the NFAT-specific complex.

since higher induction in luciferase activity was observed in transfected Jurkat cells (Fig. 6A, right panel). We were also interested in confirming these results by performing EMSA experiments with the NFAT-specific labeled probe. Results again showed no great differences between Jurkat (Fig. 6B, upper panel) and J45.01 cells (lower panel) in terms of time kinetics of NFAT translocation (Fig. 6B). Competition experiments with cold excess of NFAT oligonucleotide indicated the specificity of the signal in both cell lines (Fig. 6B, lanes 7). Our results thus suggested that the more potent induction of NFAT by PMA/Iono in J45.01 cells was not due to a difference of the time kinetic of NFAT activation as measured by the extent of NFAT translocation and NFAT-driven luciferase activity following stimulation.

Calcium mobilization in Jurkat and J45.01 cells after stimulation

Calcium mobilization was next evaluated in both cell lines after induction with the different agents. Accurate measurements of intracellular calcium release can be achieved through the use of the Indo-1 dye by calculating the ratio of calcium-bound Indo-1 over calcium-free Indo-1. As presented in the left panels of Fig. 7A, the addition of either PHA or the anti-CD3/anti-CD28 Ab combination led to a significant increase in the intracellular calcium content in a high proportion of the Jurkat cell population. With these same activators, J45.01 cells were unresponsive. However, when treated with the PMA/Iono combination, not only did both cell lines show a good response but, in addition, J45.01 cells gave a stronger response than the Jurkat parental cell line. The HPB.ALL and HPB45.0 cell lines were similarly evaluated for calcium entry (three right panels, Fig. 7A). Again, HPB45.0 cells showed higher levels of calcium mobilization than in HPB.ALL cells following PMA/Iono treatment, while these same cells were poorly responsive to either PHA or the anti-CD3/anti-CD28 Ab combination. Similar results displaying the same tendency were again obtained when we made use of these treatment settings in a third CD45-deficient cell line, H45.01, derived from the parental cell line HPB.ALL (data not shown).

To have a better mechanistic understanding of calcium modulation by CD45, we decided to analyze broken down events in intracellular calcium regulation. We first made use of EGTA, a commonly used calcium chelator. As depicted in Fig. 7B, calcium mobilization in PMA/Iono-treated HPB.ALL and HPB45.0 cells was equally dependent on the extracellular calcium source as observed by the abrupt decline in intracellular calcium content following EGTA treatment. Given that no significant differences were observed in calcium usage from extracellular sources upon PMA/Iono stimulation, more upstream events in calcium response were then investigated. Thapsigargin, a known inhibitor of the ATPase calcium pumps surfacing the cell's endoplasmic reticulum, permits a direct release of the calcium content from the endoplasmic reticulum. Upon stimulation of HPB.ALL and HPB45.0 cells with thapsigargin, no apparent differences in calcium response could be measured between these cell lines (data not shown). These results hence indicated that the decrease in calcium mobilization associated with CD45 surface expression was not linked to a direct modulation of either calcium release from intracellular stores or intake from extracellular stores. The observed events were likely to be a phenomenon dependent on membrane proximal complexes and could not be generalized to all calcium-activating pathways. However, our results obtained through calcium analysis did demonstrate that PMA/Iono-mediated intracellular mobilization was higher in CD45-negative cells.

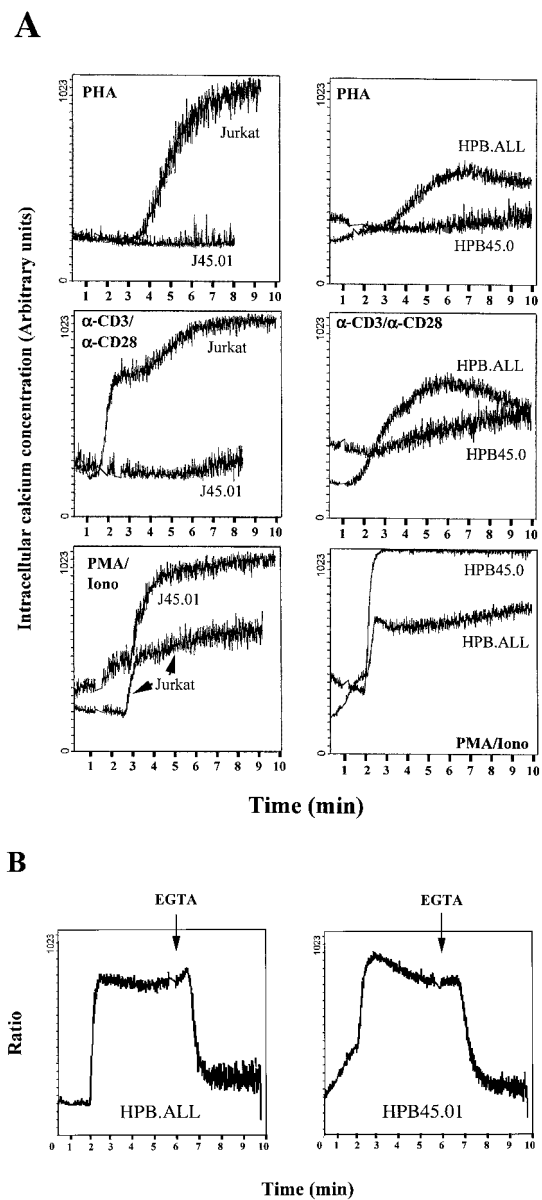


FIGURE 7. PMA/Iono-induced calcium mobilization is more active in CD45-deficient cell lines but presents a kinetic response similar to CD45-positive cells. *A*, Indo-1AM-loaded cells were incubated with the following stimulating agents: PHA (3 $\mu\text{g/ml}$), anti-CD3 (3 $\mu\text{g/ml}$)/anti-CD28 (1 $\mu\text{g/ml}$) in the presence of a goat anti-mouse IgG (5 $\mu\text{g/ml}$), or PMA (20 ng/ml)/Iono (1 μM). Jurkat and J45.01 cell responses are compared in the three left panels while HPB.ALL and HPB45.0 cell responses are compared in the three right panels. *B*, Calcium response in Indo-1AM-loaded HPB.ALL (left panel) and HPB45.01 (right panel) cells was monitored following PMA (20 ng/ml)/Iono (1 μM) stimulation for 5 min before the addition of EGTA (8 mM). Violet:blue ratios were continuously monitored and analyzed using the 1.5 version of the System 2 software (Becton-Coulter). Data are represented as the geometric mean of the violet:blue ratio (y-axis) over time (x-axis). These results are representative of two different experiments.

CD45 modulates tyrosine phosphorylation of the signaling mediators CD3 ζ and ZAP-70

Since our previous data seemed to suggest that CD45 suppressed NFAT activity following a PMA/Iono stimulation through the action of an event upstream to calcium response, we set out to investigate the phosphorylation state of early T cell signaling mediators. We first examined the phosphorylation patterns of the

well-known CD45 substrate, p56^{lck} (reviewed in Refs. 54 and 55). By means of Western blot analysis of p56^{lck}-immunoprecipitated extracts from Jurkat and J45.01 cells treated with PMA/Iono, we observed no differences in p56^{lck} protein expression and phosphorylation patterns (data not shown). We then turned our attention to the phosphorylation pattern of the ζ -chain from the CD3 TCR complex. This tyrosine-phosphorylated subunit has been shown to be an important CD45 substrate for T cell activation (56, 80). We therefore performed Western blot analysis on immunoprecipitated CD3 ζ extracts from PMA/Iono-treated Jurkat and J45.01 cells. As depicted in Fig. 8A, the tyrosine phosphorylation level of the ζ subunit (21–24 kDa) revealed by the anti-phosphotyrosine 4G10 Ab was higher in J45.01 cells in all studied time points after stimulation. This latter boost in phosphorylated ζ (ζ -P) observed in J45.01 cells was most likely due to an absence in the surface CD45 PTP activity. The reduced intensity of the ζ -P signal specifically observed in Jurkat cells stimulated for 5 min was unexpected; however, differences in TCR recycling and/or degradation induced by the different treatments might represent a potential explanation. Upon immunoblotting the same membrane with an anti-CD3 ζ chain Ab, a slightly lower band (18 kDa) appeared which corresponded to nonphosphorylated ζ and seemed to be less represented in J45.01 cells, consistent with a higher level of phosphorylated ζ subunit in this same cell line.

Given that the phosphorylation of immunoreceptor tyrosine-based activation motifs on the ζ -chain of the CD3 complex serves as a docking site for the ZAP-70 PTK (81–83). Western blot analysis of immunoprecipitated ZAP-70 from untreated or PMA/Iono-treated Jurkat and J45.01 cells was next performed. As depicted in Fig. 8B, no significant differences in the basal phosphotyrosine level of ZAP-70 were apparent in immunoprecipitated extracts from CD45-positive vs CD45-negative cells. On the other hand, CD45-negative extracts showed a substantial increase in the level of tyrosine phosphorylation of ZAP-70 at the 10-min treatment

time point. Very little differences in phosphotyrosine content of ZAP-70 were apparent in the immunoprecipitated extract from Jurkat cells at any time point following PMA/Iono stimulation. Immunoblotting with anti-ZAP-70 demonstrated that these changes in signal intensity could not be accounted by a difference in the amount of loaded ZAP-70 (Fig. 8B).

Increased NFAT1-binding activity on the HIV-1 enhancer in J45.01 cells upon PMA/Iono stimulation

Since NFAT and the HIV-1 LTR were both shown from the above results to be more activated in CD45-negative cells following PMA/Iono treatment, we next wanted to determine whether CD45 could modulate the level of HIV-1 enhancer-bound complexes. EMSA experiments were conducted with a labeled probe containing the complete enhancer region (–107/–77) of the HIV-1 LTR (Fig. 9A). We first observed that incubation of the HIV-1 enhancer probe with nuclear extracts from PMA/Iono-treated Jurkat or J45.01 cells led to the formation of a single broad signal (Fig. 9A, lanes 3 and 5). The specificity of this complex was demonstrated by the use of excess of HIV-1 enhancer or cold C/EBP oligonucleotides (Fig. 9A, lanes 6 and 7, respectively). We next thought that this signal might be consequential to an overlapping of NFAT- and NF- κ B-bound complexes. Specific competitions were hence performed with a cold NF- κ B-specific oligonucleotide to isolate the potential NFAT complex and with a NFAT-specific oligonucleotide to isolate the potential NF- κ B complex. As presented in Fig. 9A, lanes 9 and 11, competition with the NF- κ B-specific oligonucleotide led to the isolation of a band corresponding to the upper segment of the broad complex. The addition of cold excess of NFAT oligonucleotide led to the isolation of the lower complex and was thus outcompeting the upper band (Fig. 9A, lanes 13 and 15). The upper band was hence presumed to be NFAT-related and, interestingly, was found to be more intense in J45.01 cells (Fig. 9A, compare lanes 9 and 11). However, the isolated signal observed following NFAT competition (i.e., NF- κ B) was not different in terms of intensity when comparing Jurkat and J45.01 nuclear extracts (Fig. 9A, lanes 13 vs 15). To more clearly show that the lower band represented the NF- κ B complex, a supershift assay was performed with either anti-p50 or anti-p65 Abs incubated with J45.01 nuclear extracts. As depicted in Fig. 9B, both Abs induced the formation of a supershift which led to the disappearance of the lower band and the isolation of the upper band of the broad complex observed with the HIV-1 enhancer probe (compare lanes 2 and 3 with lane 1). Although the upper band seemed to diminish in intensity following the addition of the anti-p65 Ab (Fig. 9B, lane 3), this was not reproducibly observed. The anti-NFAT2 Ab did not seem to have any effect on the presence of these complexes (Fig. 9B, lane 4).

We next wanted to demonstrate the NFAT relatedness of the high molecular mass complex by supershift analysis. As presented in Fig. 9C, for both 30- and 120-min time points following PMA/Iono stimulation of J45.01 cells, the signal, which remained after competition with the NF- κ B oligonucleotide, was greatly diminished with an anti-NFAT1 Ab (Fig. 9C, lanes 5 and 12) while it was almost totally abrogated with the pan-NFAT Ab recognizing all NFAT members (lanes 6 and 13). No such effect was observed upon treatment of nuclear extracts with either anti-NFAT2 (Fig. 9C, lanes 4 and 11) or anti-NFAT4 (lanes 7 and 14) Abs, suggesting that the NFAT1 member was the most prominent constituent in the nuclear extracts of PMA/Iono-treated J45.01 cells binding to the HIV-1 enhancer region. We have also tested nuclear extracts from stimulated Jurkat cells incubated with the HIV-1 enhancer probe and have found that NFAT1 was again overrepresented in the NFAT-related bound complex (data not shown).

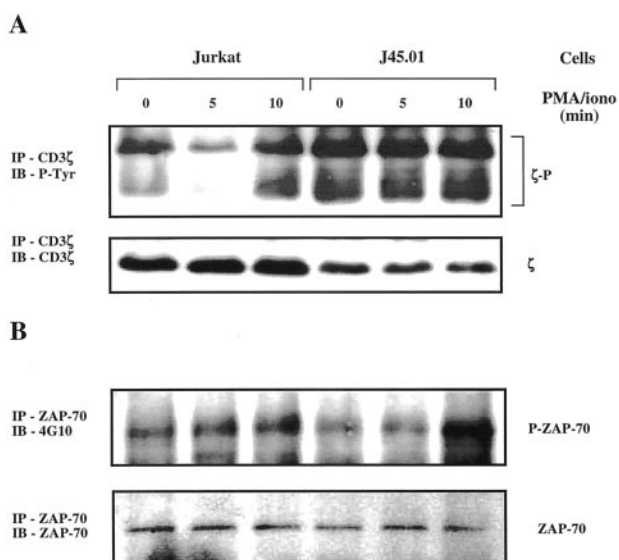


FIGURE 8. NFAT activation is mediated through the action of CD3 ζ and ZAP-70 in CD45-negative cells stimulated with PMA/Iono. Immunoprecipitations and Western blotting experiments were done on protein extracts from Jurkat and J45.01 cells (5×10^6) stimulated with PMA (20 ng/ml)/Iono (1 μ M) for 0, 5, and 10 min. *A*, PVDF membranes containing immunoprecipitated CD3 ζ were immunoblotted with anti-phosphotyrosine (upper panel) and anti-CD3 ζ (lower panel) Abs. *B*, PVDF membranes containing immunoprecipitated ZAP-70 were also immunoblotted with anti-phosphotyrosine (upper panel) and anti-ZAP-70 (lower panel) Abs.

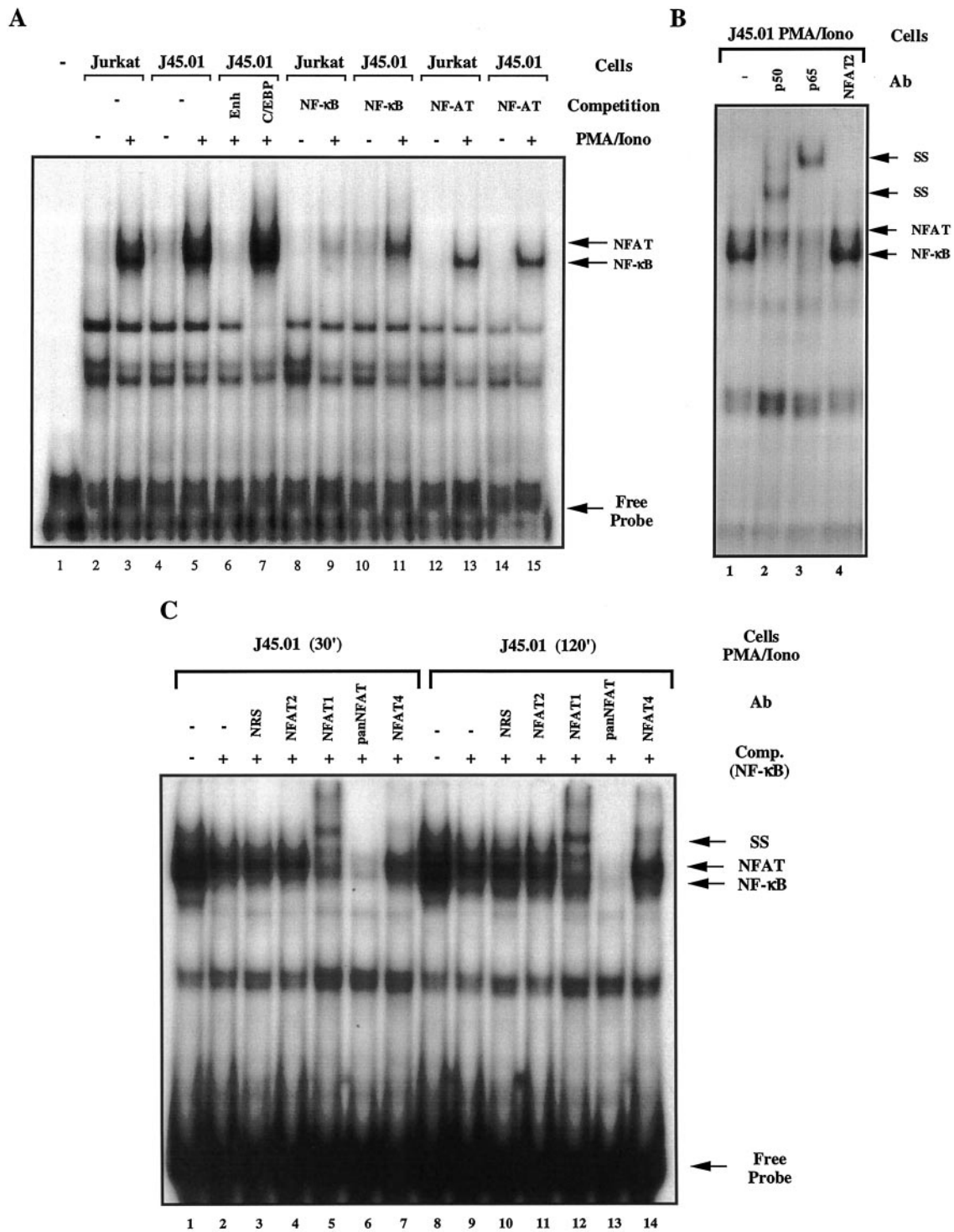


FIGURE 9. The NFAT1 member is the most prominent member binding to the HIV-1 enhancer and is increased in J45.01 cells upon PMA/Iono stimulation. **A**, Jurkat and J45.01 cells were stimulated with PMA (20 ng/ml)/Iono (1 μ M) for 1 h. Nuclear extracts from Jurkat (lanes 2, 3, 8, 9, 12, and 13) and J45.01 (lanes 4–7, 10, 11, 14, and 15) cells were incubated with an HIV-1 enhancer-labeled probe to be finally analyzed on a 4% native polyacrylamide gel. PMA/Iono treatment is indicated above each lane (–, untreated; +, PMA/Iono treated). Competition experiments were performed with 100-fold excess of either HIV-1 enhancer (lane 6), C/EBP (lane 7), NF- κ B (lanes 8–11), or NFAT (lanes 12–15) oligonucleotides. The probe only was run in lane 1. **B**, Nuclear extracts from PMA/Iono-treated J45.01 cells were incubated with the HIV-1 enhancer probe. Supershift assays were performed by preincubating nuclear extracts with anti-p50 (lane 2), anti-p65 (lane 3), or anti-NFAT2 (lane 4) Abs. **C**, Nuclear extracts from PMA/Iono-treated J45.01 cells (30 min, lanes 1–7; 120 min, lanes 8–14) were incubated with the HIV-1 enhancer probe. Supershift assays were then performed on NF- κ B-competed samples (lanes 2–7 and 9–14) by preincubating nuclear extracts with normal rabbit serum (NRS) (lanes 3 and 10), anti-NFAT2 (lanes 4 and 11), anti-NFAT1 (lanes 5 and 12), pan-NFAT (lanes 6 and 13), or anti-NFAT4 (lanes 7 and 14) Abs. Signals were resolved on a 4% native polyacrylamide gel. Arrows at the right indicate the position of the NFAT and NF- κ B complexes, the supershift (SS), and the free probe.

Thus, these results suggested that CD45-negative cells stimulated with PMA/Iono contained greater levels of the HIV-1 enhancer-bound NFAT complex and that the NFAT1 member was the most prominent member of this HIV-1 enhancer complex regardless of the cell line. These EMSA results might then explain the higher level of inducibility of the HIV-1 LTR in CD45-negative cells following PMA/Iono stimulation.

Discussion

HIV-1 LTR regulation is dependent on the presence of multiple transcription factors acting mainly through the enhancer region. Tyrosine phosphorylation events are important intracellular modifications which are known to play a crucial role in the initiation of TCR-mediated signaling and ultimately to regulate HIV-1 LTR activity. Although one study has already been addressing the contribution of the CD45 PTP on HIV-1 LTR activity at the basal level (66), we wanted to check its implication on HIV-1 LTR activity following T cell activation by different agents. From these studies, we demonstrate for the first time that CD45 negatively regulates PMA/Iono activation of the NFAT factor which we believe culminates into a lower induction of the HIV-1 LTR activity. Our results also demonstrate a potential importance for the NFAT1 member on the positive modulation of HIV-1 LTR activity targeted by CD45.

Our results first demonstrated that HIV-1 LTR activity was selectively induced in Jurkat cells over J45.01 cells when activated by TCR-dependent stimuli. This was not unexpected given the wealth of data addressing the importance of CD45 in TCR-mediated signaling (54). Unexpectedly, a more potent activation in CD45-negative cells was apparent when stimulating with the PMA/Iono inducer. We next sought the identity of the transcription factor(s) involved in this potential CD45-dependent down-regulation of the HIV-1 LTR upon PMA/Iono treatment. Two important factors are known to be crucial in HIV-1 LTR regulation: NF- κ B and NFAT, both of which act on the enhancer region of the HIV-1 LTR region (5, 46). By EMSA and luciferase assays, we have shown that NF- κ B was not differently regulated in J45.01 cells in comparison to the parental cell line following PMA/Iono treatment. This is an important issue given that Baur et al. (66) have previously demonstrated that an observed higher HIV-1 LTR basal activity in CD45-negative cell lines was consequential to higher endogenous NF- κ B-binding activity. However, in our hands, we were unable to detect any type of specific NF- κ B-binding activity in untreated J45.01 cells (see Fig. 2B). The reason for this discrepancy with the results of Baur et al. (66) is unknown but at least one other group has similarly detected no specific bands related to NF- κ B in their EMSA experiments involving J45.01 extracts (84).

When turning to the NFAT factor, we observed a stronger response in J45.01 cells for both luciferase reporter gene expression and EMSA analysis. Since NFAT frequently cooperates with AP-1 (85), we tested this other factor and found that it was more present in nuclear extracts from J45.01 cells stimulated with PMA/Iono. The increase in NFAT translocation was observed in both Jurkat- and HPB.ALL-derived CD45-deficient cell lines. These two cell settings have been known to be important in the evaluation of CD45 function on TCR-mediated T cell activation and occasionally gave contradicting results (54). The demonstration that PMA/Iono led to higher levels of NFAT translocation in the absence of CD45 for both cell settings is thus highly significant and outrules a clonal artifact of our data. CD45 regulation of NFAT activation is of important interest since it suggests a possible role of CD45 on the regulation of various genes induced by more downstream activators and containing NFAT-binding sequences. In this perspective, we have observed that the both promoters of the *IL-2* and

TNF- α genes were more greatly activated by PMA/Iono in J45.01 cells than in Jurkat cells. These results might thus suggest that the increase in *IL-2* promoter activity (at least in Jurkat cells) is mediated by a higher level of NFAT activation, confirming the results obtained by Chow et al. (86), although implication of other transcription factors such as AP-1 cannot be ruled out. However, given that a similar trend was observed in the context of the *TNF- α* promoter (where the NFAT regulatory role does not require interaction with AP-1), it is clear that a greater NFAT activation in these promoter contexts does play a role.

Analysis of the time kinetic response of NFAT activation (EMSA and luciferase activity) revealed that similar time points of NFAT activation were observed for both Jurkat and J45.01 cells. To a certain extent, we have also corroborated these data in terms of calcium mobilization induced by the PMA/Iono combination. Hence, it is likely that NFAT activation occurs through common rate-limiting steps but that the strength of the response is higher, allowing a greater level of calcineurin activation and subsequent NFAT translocation. This is thus in line with the present mechanistic understanding of NFAT activation in T cells requiring elevated calcium mobilization (31). It has also been previously suggested that a sustained calcium influx was essential for NFAT activation (31, 87). Although this might equally hold true for the higher level of NFAT activation observed in stimulated J45.01 cells, our results do not allow us to determine whether a similar conclusion can be drawn in our system. By the use of different calcium inhibitors (EGTA and thapsigargin), we have determined that the difference in calcium responses between CD45-positive and CD45-negative T cell lines was likely dependent on membrane proximal events and could not be generalized to all calcium-modulating pathways.

The mechanism underlying this CD45-dependent regulation of NFAT activation by PMA/Iono is unknown. Yet, it must be distinct from the type of induction that activates NF- κ B or, alternatively, this latter factor might not be as sensitive to the changes orchestrated by CD45 on intracellular events such as calcium release. Although CD45 has been associated with its requirement for TCR-dependent activation of T cells and the implicated cascades, several studies have indicated that, in certain contexts, CD45 could potentially inhibit the activation of specific pathways. Indeed, CD45 has been associated with a decrease in insulin signaling in a myeloma cell line (88). CD45 has also demonstrated an inhibition of both p38 mitogen-activated protein kinase activation and calcium release in Jurkat T cells induced by the addition of thrombin (89). Moreover, recently Wang et al. (71) have shown that deletion of the PTP D2 domain of CD45 led to a higher induction of calcium release as well as NFAT activation in Jurkat- and HPB.ALL-derived cell lines. It should also be stated that several groups have described a negative effect on anti-CD3-induced calcium release following Ab-mediated CD45 cross-linking (90–93). In our study, we have evaluated the importance of different proteins implicated in TCR-mediated signaling through the use of Western blot analysis to identify and elucidate mechanisms underlining the CD45-mediated suppression of NFAT activity following PMA/Iono treatment. Our results suggest that CD3 ζ chain dephosphorylation by CD45 could be important in the observed differences in the level of NFAT activation following PMA/Iono stimulation. A higher tyrosine phosphorylation level of the CD3 ζ chain present in CD45-negative cell lines might account for the observed induced tyrosine phosphorylation of ZAP-70. Alternatively, the absence of CD45 might directly lead to an increase in ZAP-70 phosphorylation. In either condition, higher ZAP-70 PTK activity could cooperate exclusively with more downstream effectors (such as PMA/Iono) on the activation of NFAT through its direct action on phospholipase

$\text{C}\gamma 1$ (PLC $\gamma 1$) activity required for the induction of calcium mobilization (reviewed in Ref. 55). Such an increase in PLC $\gamma 1$ activity might also be a determinant for the higher level of AP-1 complex observed in CD45-negative cells.

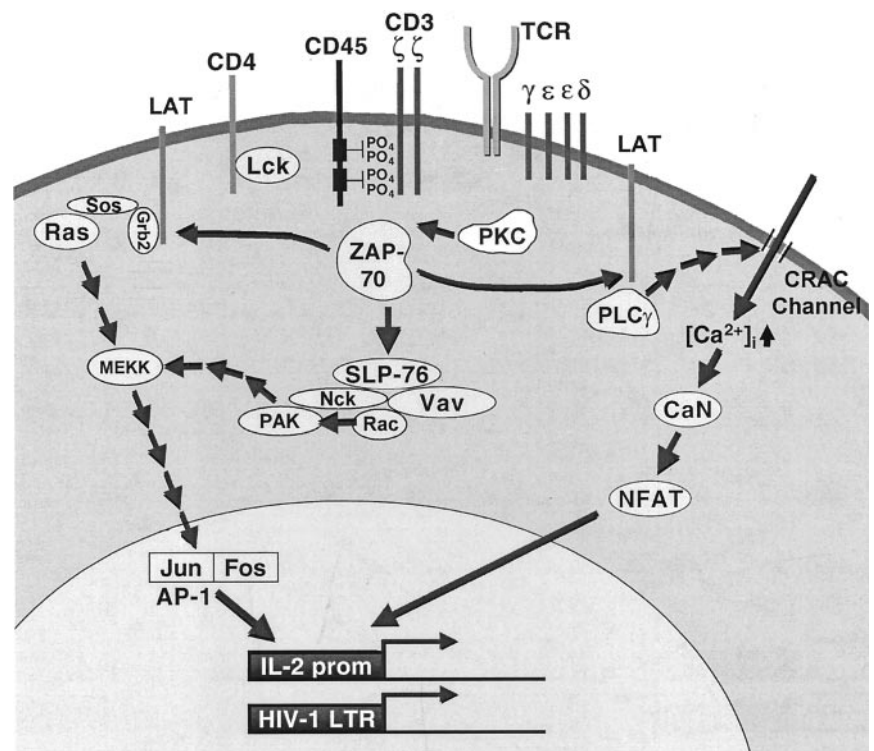
Based on our results, we further suggest that PMA/Iono activation of the ZAP-70 PTK might be occurring through targeting of the TCR complex by protein kinase C (PKC) itself (Fig. 10). Such a link between PKC and the TCR complex has been previously reported in the context of PKC-induced TCR recycling (94–96), a phenomenon which was observed to be CD45 and $\text{p}56^{\text{Lck}}$ independent. Interestingly, a similar negative role for CD45 has been recently inferred on PMA-induced differentiation of monocytic cells (97). The direct and specific mechanism by which PMA could induce cascade signaling through early events of the CD3-TCR complex leading to NFAT activation still remains unknown. PKC is known to be recruited to the membrane once activated by PMA and could consequently act on potential membrane-proximal substrates that could induce TCR-mediated signaling pathways. Hence, according to our model (Fig. 10), the activation of PKC by PMA would initiate a cascade through the targeting of the TCR complex. The activation of the PTK ZAP-70 might then be dependent on a primed CD3 ζ chain such as in the hyperphosphorylated version in CD45-negative cell lines. The phosphorylation and activation of ZAP-70 would then lead to PLC $\gamma 1$ activation, which would, in turn, permit intracellular calcium release and entry via the Ca^{2+} release-activated Ca^{2+} pumps. The activation of both PKC and the ras pathway by ZAP-70 would also lead to more potent activation of the AP-1 complex. These observations indicate that CD45 acts at different steps through its positive and negative regulatory role in T cell activation.

Our study has also permitted us to assess the type of complexes which are bound to the HIV-1 enhancer region directly from stimulated extracts. With different competition conditions, NFAT-specific bands were observed to be more intense in extracts from stimulated J45.01 cells than in Jurkat extracts whereas NF- κ B did not seem to be affected. Supershift experiments indicated that nu-

clear extracts from PMA/Iono-stimulated Jurkat and J45.01 cells prominently contained the NFAT1 member in the HIV-1 enhancer-bound complex. It is important to point out that we have previously observed that competition experiments with NF- κ B did not lead to removal of any NFAT member-related signals bound to a NFAT probe (data not shown). Nonetheless, to assure that this overrepresentation of the NFAT1 member in the HIV-1 enhancer-associated NFAT complex was not due to competition of the other NFAT members by the NF- κ B oligonucleotide, we have performed supershift experiments with one or two Abs consisting of either anti-p65-treated samples vs anti-p65/anti-NFAT-treated samples. Comparing to the anti-p65 Ab-treated signal, we again confirmed that the anti-NFAT1 Ab was the most effective Ab to reduce the intensity of the NFAT-related signal (data not shown).

The implication of NFAT in HIV-1 replication and HIV-1 LTR activity through the HIV-1 enhancer region has already been strongly suggested in several studies (18–20, 46, 47). However, we were surprised to find that most of the NFAT-binding activity on the HIV-1 enhancer was accounted by the NFAT1 family member. Although Jurkat is known to express different NFAT family members (NFAT1 through NFAT4) (26, 85), recent studies have indicated that the different NFAT members were not acting in a similar fashion on the HIV-1 LTR activity. Indeed, while Kinoshita et al. (46) have demonstrated a positive modulation of HIV-1 LTR activity by the NFAT2 member, Macián and Rao (47) have rather proposed a negative regulatory role of the NFAT1 member through what was hypothesized to be a competition with NF- κ B for the binding of the HIV-1 enhancer region. Although these latter results seem discordant with our own, it should be stated that our analysis is based on induced signaling pathways which lead to translocation of naturally occurring levels of both intracellular NFAT and NF- κ B. Supporting our data, a study by Cron et al. (21) has indicated that NFAT1 and NFAT2 were both acting positively on HIV-1 LTR activity in primary CD4 $^{+}$ T cells. The study of Macián and Rao (47) has been mainly focusing on

FIGURE 10. Proposed model defining mediators and signaling in PKC-mediated NFAT activation. Activation of PKC by PMA would directly act upon TCR-related signaling events. Subsequent ZAP-70 activation would be highly dependent on the presence of an hyperphosphorylated CD3 ζ (present in CD45-deficient cell lines). Activated ZAP-70 could then, in turn, promote downstream events leading to mitogen extracellular-regulated kinase kinases (MEKK) and PLC $\gamma 1$ activity, which would result in AP-1 activation and calcium release, respectively. The higher level of activation of the NFAT factor in CD45-negative cell lines would thus lead to a stronger induction of HIV-1 LTR and *IL-2* gene transcription. Multiple arrows indicate a multistep process linking key mediators.



transient transfection experiments with an NFAT1-expressing vector and might consequently differ from HIV-1 LTR regulation dependent on the normal endogenous NFAT pools. In addition, most of the studies from Macián and Rao were based on the use of single NF- κ B repeat-containing plasmid which might not properly address the potential synergistic action of NFAT and NF- κ B on the HIV-1 LTR activity. One other issue that needs to be drawn to the attention relates to the observed binding of the NFAT2 member to the HIV-1 enhancer previously described in stimulated Jurkat cells (46). It is possible that the NFAT1 member normally outcompetes the binding of NFAT2; however, it is also possible that a second factor could greatly improve the binding of NFAT2 to the HIV-1 enhancer which we might not be able to assess through EMSA experiments with total nuclear extracts. In this perspective, NFAT2 (or other NFAT family members) might also be important in HIV-1 regulation following PMA/Iono stimulation when bound to the HIV-1 enhancer through a cooperative interaction with this other factor. This would in fact be reminiscent of the previously described cooperative binding between NFAT family members and AP-1 for the NFAT binding site of the *IL-2* promoter region (23). We are presently examining the impact of the different NFAT family members on overall HIV-1 LTR activity.

Since previous studies have suggested that HIV-1 infection impaired CD45 cell surface expression or PTP activity (66, 98, 99), it can be postulated that if a similar *in vivo* regulation of NFAT activation by CD45 occurs, it hence follows that CD4⁺ T cells impaired in their CD45 PTP activity might be more prone to HIV-1 LTR activation upon TCR-independent stimulation. For instance, pathways activated by RANTES or the transmembrane activator and cAML interaction, CD43, and CD2 cell surface molecules have all been shown to culminate into calcium mobilization and NFAT activation in T cells (100–103) and might be such examples. Our results have hence demonstrated that CD45 negatively regulates NFAT and HIV-1 LTR activation following PMA/Iono treatment. This finding might be indicative of the negative role played by CD45 on certain segments of the TCR signaling pathway such as CD3 ζ and ZAP-70 activity. Additional studies are presently in progress in our laboratory to gain a more detailed description of this potential interrelationship among PKC, TCR, and CD45 leading to differences in calcium mobilization consequently resulting in a higher level of NFAT and HIV-1 LTR activation.

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