

Grb3-3 Is Up-regulated in HIV-1-infected T-cells and Can Potentiate Cell Activation through NFATc*

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The MAPK pathway is required for T-cell activation; however, its role in modulating T-cell function following human immunodeficiency virus type 1 (HIV-1) infection is poorly understood. In this report, we investigated whether Grb3-3, an isoform of the Grb2 (growth factor receptor-bound protein-2) adaptor molecule that is associated with the MAPK pathway, could be involved. We found that Grb3-3, but not its isoform Grb2, is markedly up-regulated in CD4⁺ peripheral blood mononuclear cells derived from either *in vitro* HIV-1-infected cultures or HIV-1-infected human subjects. Analysis of HIV-1 gene products indicated that Tat and Nef, both of which have been implicated in modulating T-cell function, can independently induce expression of Grb3-3. By using NFAT/AP-1, AP-1, or NFAT reporter assays, we found that Grb3-3 can potentiate NFAT (but not AP-1) promoter activity in Jurkat T-cells upon engagement of the T-cell receptor and CD28 co-receptor. In addition, potentiation of NFAT by Grb3-3 is substantially suppressed by MEKK1, a kinase that may play an important role in retaining NFAT in the cytoplasm, and by cyclosporin A. Finally, we also found that Grb3-3 potentiates HIV-1 long terminal (LTR) repeat promoter activity following T-cell receptor stimulation, an effect that can be largely suppressed by cyclosporin A. Taken together, this study indicates that Grb3-3 is a cellular factor that can be up-regulated by HIV-1. In addition, Grb3-3 can also function as a positive factor for T-cell activation and, in doing so, may aid in establishing an intracellular environment that can optimally support HIV-1 replication.

(PBMCs) cannot be infected efficiently unless prestimulated with activation agents such as phytohemagglutinin and anti-CD3 antibody, whereas treatment of latently infected cells with a variety of activation agents can markedly enhance viral particle production (1–6). These observations indeed point to a close relationship between viral replication and T-cell activation.

T-cell activation initiates upon engagement of the T-cell antigen receptor (TCR or CD3) and additional co-receptors such as CD28 by extracellular molecules presented by antigen-presenting cells (7–9). Following receptor engagement, multiple intracellular signals are activated, leading to increased transcription of a variety of activation-associated genes encoding immunoresponsive cytokines (10–13). Previous studies indicate that transcription of these cytokine genes can be drastically inhibited by immunosuppressive drugs such as cyclosporin A (CsA) and FK506 because these drugs suppress the functional activity of calcineurin (14). Calcineurin is a calcium-dependent phosphatase that dephosphorylates NFAT (nuclear factor of activated T-cells), thereby enabling the latter to translocate into the nucleus and to bind to the enhancer region of the cytokine gene (13–16). More important, the fact that blocking nuclear import of NFAT can shut off transcription of these genes strongly indicates that NFAT is one of the essential transcription factors for activation of these cytokine genes (15–20).

Several groups have investigated potential roles of NFAT in HIV-1 replication and/or pathogenesis, especially interaction of the viral protein Tat with NFAT (21–24). It is likely that different members of the NFAT family may have distinct effects on HIV-1 replication (22–24) and, in the case of NFATc, may influence viral replication at multiple levels. Because signaling molecules in the MAPK pathway are known to be involved in NFAT/T-cell activation and to be activated in HIV-1 infection (25–29), we investigated whether an isoform of Grb2 (growth factor receptor-bound protein-2) (30) named Grb3-3 (31) could be involved in this process. Grb2 is an adapter protein that is known to participate in a variety of signal transduction pathways, including the MAPK pathway and T-cell activation (30–37). Structural analysis indicates that Grb2 consists of an SH2 domain flanked by two SH3 domains and that Grb3-3 is structurally similar to Grb2, except that it has a truncated SH2 domain (deleted in exon III, amino acids 60–100 of Grb2) but retains two intact SH3 domains (30, 31). As compared with Grb2, the functional activities of Grb3-3 remain largely unknown, except that overexpression can cause apoptosis in NIH3T3 cells (31).

In this report, we found that Grb3-3, but not its isoform Grb2, is markedly up-regulated in CD4⁺ PBMCs derived from

Productive HIV-1¹ replication of primary CD4⁺ T-cells requires that these cells be in an active state. For example, in tissue culture, quiescent peripheral blood mononuclear cells

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¹ The abbreviations used are: HIV-1, human immunodeficiency virus type 1; PBMC, peripheral blood mononuclear cell; TCR, T-cell receptor; CsA, cyclosporin A; MAPK, mitogen-activated protein kinase; MEKK1, MAPK/extracellular signal-regulated kinase kinase kinase-1; LTR, long terminal repeat; mAb, monoclonal antibody; GST, glutathione S-transferase; PCR, polymerase chain reaction; PMA, phorbol 12-myristate 13-acetate; JNKs, c-Jun NH₂-terminal kinases.

either *in vitro* HIV-1-infected cultures or HIV-1-infected patients. Analysis of HIV-1 gene products indicated that expression of Grb3-3 can be independently induced by Tat and Nef, two early viral proteins that are known to modulate T-cell function (38–45). By using NFAT/AP-1, AP-1, or NFAT reporter assays, we found that Grb3-3 can potentiate NFAT (but not AP-1) promoter activity in Jurkat T-cells upon engagement of the TCR and CD28 co-receptor. In addition, potentiation of NFAT by Grb3-3 is substantially suppressed by MEKK1*, a kinase that may play a critical role in opposing NFAT translocation into the nucleus (46), and by CsA. Finally, Grb3-3 stimulates HIV LTR promoter activity upon engagement of CD3 and CD28 receptors, an effect that can also be inhibited by CsA. Our findings demonstrate that Grb3-3 is a cellular factor that is markedly up-regulated by HIV-1 infection and may act as a positive regulator for both T-cell activation and HIV-1 replication through its actions on NFATc.

EXPERIMENTAL PROCEDURES

Patients—The patient blood samples were obtained at Rothschild Hospital (Dr. W. Rozenbaum) from seropositive persons from November 1993 to October 1994. Heparinized blood samples were collected after informed consent of HIV-1-seropositive ($n = 31$) and HIV-seronegative ($n = 10$) individuals. For 25 of the HIV-1-seropositive patients, clinical data were available. Using the Centers for Disease Control classification, the clinical disease status of the 25 patients was as follows: Group II in two cases, Group III in three cases, and Group IV in 20 cases. Twenty-three patients were under treatment at the time of the study; however, because of the years that these samples were obtained, none of these patients were receiving highly active anti-retroviral therapies. Six patients were seropositive for HIV p24 antigen. The HIV-1-seronegative individuals were laboratory personnel.

Generation of mAbs against Grb2/Grb3-3 and Western Blot Assay—GST-Grb3-3 and a 13-mer peptide, EMKPHFPFGNDVQ, spanning the junction between the first SH3 domain and the truncated SH2 domain of the *grb3-3* gene (31) and covalently coupled to keyhole limpet hemocyanin, were used separately to immunize female BALB/c mice. Hybridoma 3B5 recovered from the GST-Grb3-3-immunized mice bound Grb2 and Grb3-3 in enzyme-linked immunosorbent assay, Western blotting, and immunoprecipitation, whereas hybridoma 4F2 recovered from the keyhole limpet hemocyanin-coupled 13-mer-immunized mice bound specifically to Grb3-3 alone in all three assays.

Western blot assays were performed as follows. Equal amounts of whole cellular proteins were fractionated on SDS-12.5% polyacrylamide gel, followed by transferring the samples to a nitrocellulose filter. mAb 3B5 against Grb2/Grb3-3 was used as the primary antibody. Finally, peroxidase-conjugated goat anti-mouse IgG (Sigma) was added for a further incubation of 1 h at room temperature. The results were analyzed using an Amersham Pharmacia Biotech ECL kit.

Detection of HIV-1 viral proteins in cells transfected with various expression vectors was also performed in Western blot assays as described above. Human sera from HIV-1-positive individuals were used to detect envelope protein. Rabbit anti-Rev antibody was kindly provided by Dr. Alan Cochrane (University of Toronto). Anti-Tat antibody was a gift from Dr. Bryan Cullen (Duke University). Anti-Nef antibody was obtained from Dr. Ron Swanstrom (University of North Carolina). Anti-Vif antibody was a gift from Dr. Dana Gabuzda (Dana-Farber Cancer Institute). Hemagglutinin-tagged Vpr (from Dr. Nat Landau, Salk Institute) was detected using anti-hemagglutinin monoclonal antibody purchased from Babco (Berkeley, CA).

Analysis of Grb3-3 and Grb2 mRNAs and Proteins in Patient PB-MCs—Selection of PCR primers and optimization of reaction conditions such as β -actin controls and titration curves have been described previously (31).

Plasmids and Proviral DNA—pSV-Env and pSV-Rev were obtained from Dr. Joseph Sodroski (Dana-Farber Cancer Institute). pcDNA-Vif and pSV-Nef were obtained from Dr. Dana Gabuzda. pcDNA-Vpr (with a hemagglutinin tag) was a gift from Dr. Nat Landau (47). pNL4-3 (an infectious HIV-1 proviral clone) was obtained from the National Institutes of Health AIDS Reagents and Resource Repository. pcDNA-Tat was obtained from Dr. Bryan Cullen. The cDNA clones encoding Grb2 and Grb3-3 were amplified by PCR from template pGST-Grb2 and pGST-Grb3-3 using *Pfu* polymerase (31) and were each cloned into pSVL (Amersham Pharmacia Biotech). The 3xNFAT/AP-1-LUC reporter with three binding sites for NFAT/AP-1 was obtained from Dr.

Steven Burakoff (Dana-Farber Cancer Institute). The 3xAP-1-LUC and 3xNFAT-LUC reporters with three binding sites for AP-1 and NFAT, respectively, were obtained from Dr. Chris Rudd (Dana-Farber Cancer Institute). The pNFATc eukaryotic expression vector was a generous gift from Dr. Gerald Crabtree (Stanford University) (48). The eukaryotic expression vectors pSV-Grb2 and pSV-Grb3-3 were as described (31). Constitutively activated MEKK1* (the C-terminal catalytic domain of MEKK1 (301–672)) was kindly provided by Dr. Jacques Pous-ségur (UMR 6543, CNRS, Université de Nice, Nice, France). It was cloned into the pcDNA3 eukaryotic vector (49).

Cell Transfection, Promoter-Luciferase Assay, and Detection of NFAT in the Nucleus—Jurkat cells (5×10^6) were transfected with DNA constructs using LipofectAMINE (Life Technologies, Inc.) as transfection mediator following the manufacturer's instruction. Typically, in each transfection, 2 μ g of luciferase reporter and 4 μ g of DNA constructs expressing exogenous proteins were used (see "Results"). In all transfection experiments, 1 μ g of the β -galactosidase expression vector pcDNA-LacZ (Invitrogen) was cotransfected, and triplicate transfections were used to standardize transfection efficiency. Empty vectors were used to bring the total amount of DNA to 10 μ g. Twenty-four hours after transfection, the cells were activated for 5–8 h with various stimuli. Usually, PMA (Sigma) and ionomycin (Calbiochem) were used at 10 nM and 0.5 μ M, respectively. Mouse monoclonal antibody against Sp1 (sc-420) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal antibodies against CD3 and CD28 were purchased from Pharmingen (San Diego, CA) and used at 1 μ g/ml. Rabbit anti-mouse IgG was obtained from Sigma and used at 2 μ g/ml to cross-link anti-CD3 and anti-CD28 antibodies 2 h before luciferase assays (Promega kit) were performed. Data are represented as -fold increase in luciferase activity compared with the empty vector control.

For detection of NFATc in the nucleus, we carried out nuclear protein extraction essentially as described (50). Twenty micrograms of total nuclear extracts were subjected to Western blot analysis using mouse monoclonal antibody against NFATc (sc-7294, Santa Cruz Biotechnology). As an internal control, transcription factor Sp1 was also probed using anti-Sp1 antibody (sc-420) purchased from Santa Cruz Biotechnology.

RESULTS AND DISCUSSION

Several lines of evidences prompted us to investigate possible relationships between Grb3-3 and HIV-1 infection. (i) Signaling molecules of the Ras/MAPK pathways are known to be activated following HIV-1 infection (25–29). (ii) Grb2 and its isoform Grb3-3 are molecules associated with the Ras/MAPK pathways (30, 31). (iii) T-cell activation is required for productive HIV-1 replication, and Grb2 is known to play an important role in T-cell activation (2–6, 32–37). We initiated our studies by analyzing Grb3-3 expression in CD4⁺ PBMCs following HIV-1 infection.

Up-regulation of Grb3-3 Expression in CD4⁺ Peripheral Blood Lymphocytes Infected with HIV-1—CD4⁺ PBMCs were stimulated with phytohemagglutinin (1 μ g/ml) for 3 days and subsequently infected with HIV-1 (strain NL4-3, 5 ng of p24/10⁷ cells) (51). As expected, productive infection by HIV-1 was observed in CD4⁺ cells, with a peak of virus production ~1 week after infection (Fig. 1A). We next analyzed Grb3-3 protein expression in these same cultures by Western blotting. As shown in Fig. 1B, Grb3-3 was not detected, and there was little change in Grb2 in uninfected cells during the entire 9-day culture period. Infection of CD4⁺ PBMCs with HIV-1 caused an ~2-fold increase in Grb2 levels; however, in striking contrast, there was a steady increase in the level of Grb3-3 (Fig. 1C), and densitometry scanning of these protein bands revealed an 80-fold increase in Grb3-3 expression levels as compared with only a 2-fold increase in Grb2 expression levels in a 9-day infection period (Fig. 1D). Similar results were obtained in Jurkat T-cells infected with HIV-1 (data not shown). These results indicate that Grb3-3 is up-regulated by HIV-1 infection *in vitro*.

Detection of Overexpressed Grb3-3 mRNA and Proteins in HIV-1-infected Human Subjects—To extend our investigation on the relationship between HIV-1 infection and Grb3-3 up-regulation, we evaluated the expression of Grb2 and Grb3-3

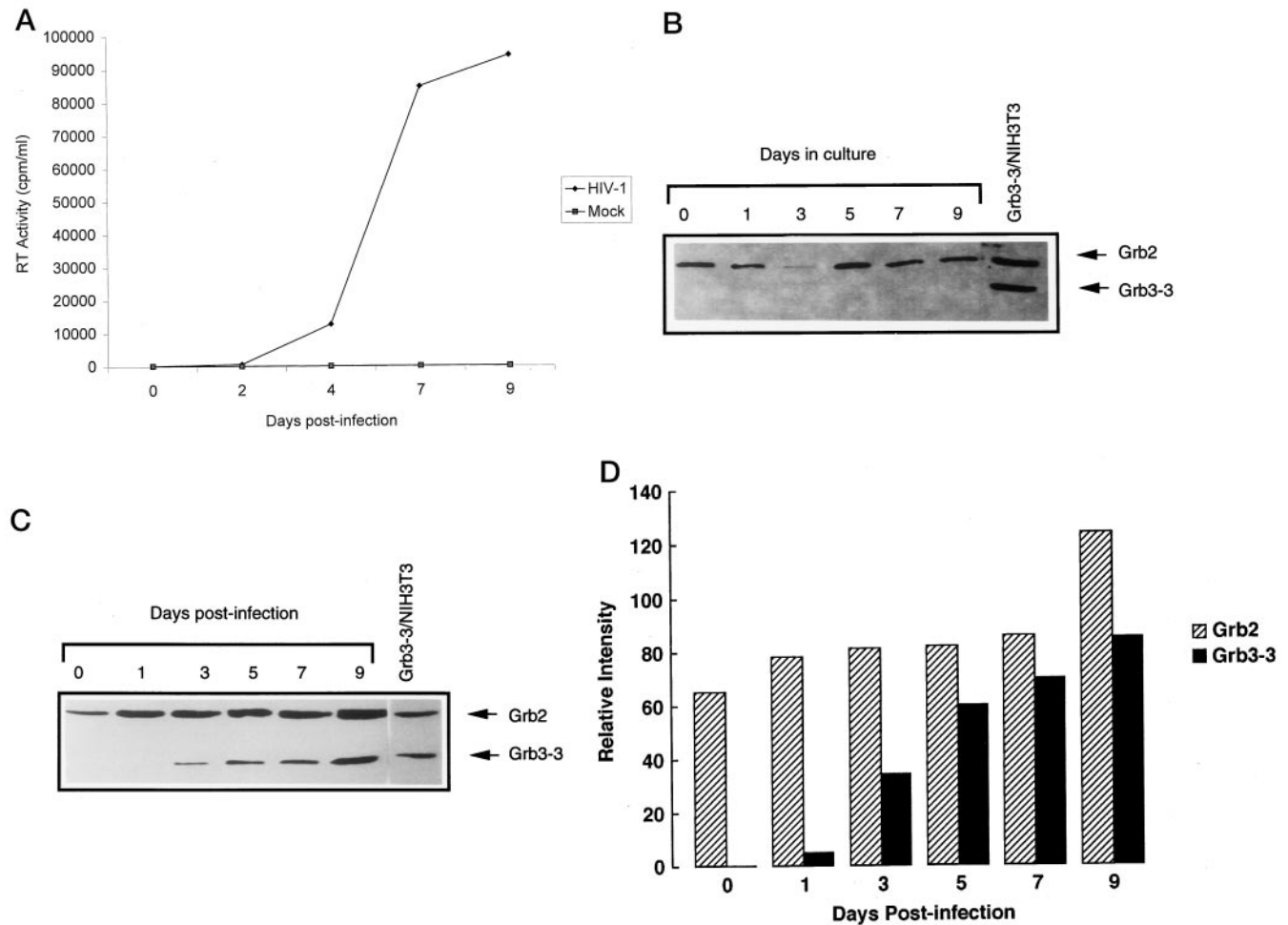


FIG. 1. Up-regulation of Grb3-3 in CD4⁺ PBMCs following HIV-1 infection. A, infection of CD4⁺ PBMCs. Viral stocks were prepared by transfection of CD4⁺ Jurkat cells with pNL4-3 proviral DNA as described under "Experimental Procedures" (51). CD4⁺ PBMCs were isolated from healthy donors using Ficoll-Hypaque gradient centrifugation (Amersham Pharmacia Biotech), followed by selection on OKT4-coated flasks (Applied Immune Sciences, Inc., Santa Clara, CA) according to instructions from the manufacturer. The selected CD4⁺ mononuclear cells were stimulated with phytohemagglutinin at a concentration of 1 μ g/ml for 3 days. Cells (10^7) were infected with 5 ng of HIV-1 p24 for 3 h, followed by washing three times with phosphate-buffered saline. The cells were then resuspended in fresh RPMI 1640 medium and 10% fetal calf serum. Supernatants were collected periodically for reverse transcriptase (RT) assays (51). \blacklozenge , HIV-1-infected cells; \blacksquare , mock-infected cells. B–D, determination of Grb2 and Grb3-3 protein expression. Cells were harvested for isolation of total cellular protein. Equal amounts of protein were fractionated on SDS-12.5% polyacrylamide gel, followed by transfer to a nitrocellulose filter. Detection of the protein was achieved by Western blotting using mAb 3B5, which binds to both Grb2 and Grb3-3. B, Grb2 and Grb3-3 protein expression in uninfected cells (mock infection). C, Grb2 and Grb3-3 protein expression in HIV-1-infected cells (CD4⁺ PBMCs infected with HIV-1 (strain NL4-3)); D, quantitative densitometry of Grb2 and Grb3-3 protein levels in CD4⁺ mononuclear cells during HIV-1 infection (for C). The Western blot shown in C was subjected to quantitative densitometry measurements. The values shown represent relative density measurements over the 9-day experiment and are expressed as relative density units.

mRNAs in PBMCs obtained from 31 HIV-1-seropositive individuals and compared them with mRNAs from 10 HIV-1-negative healthy donors using reverse transcription-PCR assay. Selection of PCR primers and optimization of reaction conditions have been described previously (31). As a control to ensure that the same amounts of cDNA were used in all experiments, β -actin-encoding cDNA was amplified by PCR with specific oligonucleotides (31). A titration curve was established to quantitate the ratios of Grb3-3 to Grb2. PCR products were analyzed on 1.5% agarose gels stained with 0.5 mg/ml ethidium bromide and visualized under UV light. As is shown in Table I, in PBMCs from all seronegative donors, Grb3-3 mRNA was barely detectable, whereas Grb2 mRNA levels were constant. In contrast, PBMCs from all seropositive individuals displayed an increased level of Grb3-3. In two cases, the same amount of Grb3-3 and Grb2 mRNAs was detected (patients 13 and 15); in 29 cases, the ratio of Grb3-3 to Grb2 was found to be >1 . Of the 29 cases, seven patients had undetectable Grb2 mRNA, but elevated Grb3-3 mRNA levels (patients 16–22), and seven pa-

tients had lower Grb2 mRNA levels compared with those of the control subjects, but increased levels of Grb3-3 (patients 23–29). Nine patients had higher Grb3-3 levels, but normal (control subjects) levels of Grb2 mRNA (patients 7–12, 14, 30, and 31), whereas four patients had Grb3-3 mRNA levels greater than control Grb2 mRNA levels (patients 1 and 4–6). Only two seropositive patients had a ratio of Grb3-3 to Grb2 mRNA of <1 . Taken together, despite variations in the Grb3-3/Grb2 mRNA ratios, elevated Grb3-3 mRNA levels were consistently detected in the HIV-1-infected subjects, although the available data do not allow one to establish a clear relationship between the clinical stages of disease and the value of the Grb3-3/Grb2 mRNA ratios. A representative sample of these experiments is shown in Fig. 2A. It is noteworthy that Grb2 was observed in all samples from healthy donors, whereas in $\sim 24\%$ of the patient samples, we failed to detect Grb2 for unclear reasons (Table I). A correlation between HIV-1 RNA copy number and Grb3-3/Grb2 ratios was not performed because at the time when these studies were initiated, analyses of viral loads were

TABLE I
Detection of Grb3-3 mRNA in PBMCs from HIV-1-seropositive and HIV-1-seronegative individuals by reverse transcription

Patient identification	Mortality ^a	Sex	Age	CDC ^b staging	p24 antigen	Grb3-3/Grb2 mRNA ^c
	%		years		pg/ml	
1	1.90				n	>1
2	0					<1
3	0					<1
4	0					>1
5	5.80					>1
6	2.80					>1
7	0.60	M	34	III	0	>>1
8	0	M	39	IVC2	0	>>1
9	0.02	M	51	IVC1, IVD	0	>>1
10	0	M	55	IVD	0	>>1
11	0	F	31	IVC1	0	>>1
12	0	F	26	IVC1	80	>>1
13	0.05	M	46	IVC2	0	<1
14	0	M	55	IVC2	0	>>1
15	0	M	56	IVD	7	<1
16	0	M	37	III	0	>>1 Grb2 = 0
17	0	F	30	IVC1	87.4	>>1 Grb2 = 0
18	0	M	31	IVD	0	>>1 Grb2 = 0
19	0	M	40	IVC2	0	>>1 Grb2 = 0
20	0	M	31	II	0	>>1 Grb2 = 0
21	0	M	33	IVC2	0	>>1 Grb2 = 0
22	0	M	44	IVC1	9.7	>>1 Grb2 = 0
23	5.20	M	30	III	21.5	>>1 Grb2 low
24	10	F	63	IVC2, IVE	0	>>1 Grb2 low
25	0	M	47	IVC2	7	>>1 Grb2 low
26	<1	M	31	IVC2	0	>>1 Grb2 low
27	3.50	M	49	IVC2	40.6	>>1 Grb2 low
28	1	M	37	IVD, IVC1	0	>>1 Grb2 low
29	12	F	30	IVC2	0	>>1 Grb2 low
30	3	F	31	IVC1	0	>>1
31	1.90	F	40	II	0	>>1

^a Mortality as determined by trypan blue assay after cell collection.
^b Centers for Disease Control.
^c See Fig. 2A for details of reverse transcription-PCR.

not routinely performed (see “Experimental Procedures”).

Grb3-3 protein expression was also analyzed by Western blotting of protein samples available from a limited number of HIV-1-seropositive individuals. The results are shown in Fig. 2B. *Lane 1* shows a negative control (NIH3T3 cells transfected with the empty vector), and *lane 2* shows a positive control (NIH3T3 cells transfected with a Grb3-3 expression construct), whereas *lane 3* represents a seronegative healthy donor. Clearly, Grb3-3 protein was detected in the samples from all five patients (*lanes 4–8*). Taken together, these data indicate that in seronegative individuals, Grb3-3 protein expression is very low or undetectable, whereas HIV-1 infection causes up-regulation of Grb3-3 *in vivo*, an observation that is in general agreement with the *in vitro* HIV-1 challenge data.

Viral Proteins Tat and Nef Can Independently Induce Grb3-3 Expression—We next determined which viral component(s) was responsible for up-regulation of Grb3-3 using constructs expressing various viral proteins. To this end, Jurkat T-cells were transfected with various plasmids expressing HIV-1 Tat, Rev, Vif, Vpr, Env, or Nef. Cells were harvested 72 h post-transfection and lysed for extraction of total cellular proteins for Western blot analyses. We first confirmed, in a Western blot assay, that these viral protein expression constructs indeed produced corresponding viral proteins in the transfected Jurkat cells (Fig. 2C). We next investigated which viral protein(s) was responsible for up-regulation of Grb3-3. As is shown in Fig. 2D, transfection of the *tat* and *nef* genes resulted in an increased expression of Grb3-3 (*lanes 7 and 8*), but no up-regulation of Grb3-3 was detected in cells transfected with the empty vector, pSV-Env, pSV-Rev, pcDNA-Vif, or pcDNA-Vpr (*lanes 3–6*, respectively). The level of expression of Grb3-3 was dose-dependent on the amount of transfected Tat and Nef expression plasmids (data not shown). Also shown in Fig. 2D is

the positive control (*lane 1*), in which Jurkat T-cells were transfected with a Grb3-3 expression plasmid. These results indicate that two of the earliest viral proteins, Tat and Nef, can independently induce Grb3-3 expression in Jurkat T-cells. Although Tat and Nef are known to participate in a variety of cellular activities, including interaction with MAPK pathways and modulation of T-cell functional activities (38–45), our studies show that they share some unknown but common cellular activation pathway(s) that can lead to alternative splicing of Grb2. Since Tat and Nef can also act as positive factors for T-cell activation and viral replication through activation of cell proliferation pathway(s) and/or by lowering the T-cell activation threshold (27–40, 42–45, 52–57), it was of particular interest to evaluate cellular factors that may contribute to the virus-induced cell activation. Since Grb2 is known to be involved in T-cell activation, a role of Grb3-3 in T-cell activation was next investigated (see below).

Grb3-3 Potentiates NFAT and HIV-1 LTR (but Not AP-1) Promoter Activities in Jurkat T-cells—Among the transcription factors, NFAT is indispensable for T-cell activation (15, 16). A critical step in this activation pathway is that cytoplasmic phosphorylated NFAT must be dephosphorylated by calcineurin. The dephosphorylated NFAT is then able to translocate into the nucleus (11, 13, 16). To analyze the role of Grb3-3 in T-cell activation, Jurkat T-cells were cotransfected with the 3xNFAT/AP-1-LUC reporter (containing three tandem repeats of the NFAT/AP-1-binding site) and either Grb3-3 or the empty vector, followed by treatment with either anti-CD3/anti-CD28 antibodies or chemical stimuli such as PMA and/or ionomycin. As is shown in Fig. 3A, in the absence of stimulation with exogenous stimuli, Grb3-3 was not able to affect NFAT/AP-1 activity. However, Grb3-3 caused an ~10-fold increase in luciferase activity as compared with the vector control following

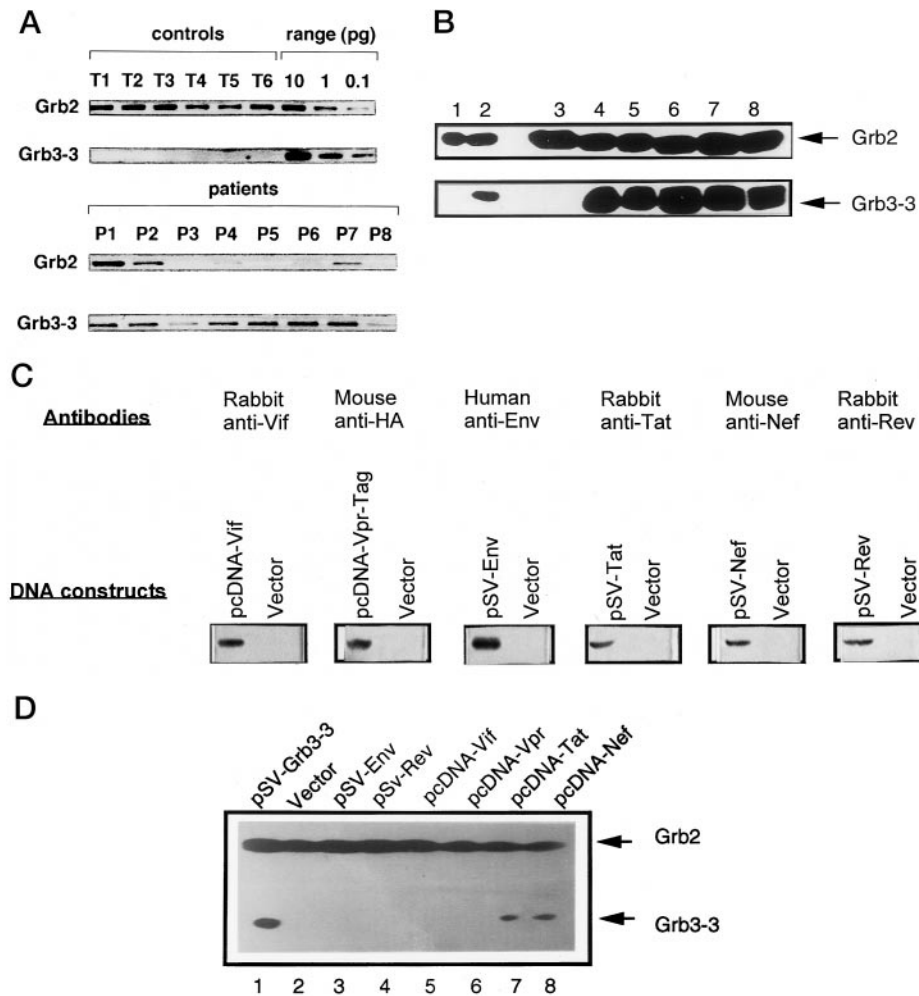


FIG. 2. Detection of Grb3-3 and Grb2 mRNA and protein expression. A, reverse transcription-PCR of Grb2 and Grb3-3 was performed as described previously (31) using total RNAs isolated from PBMCs of HIV-1-infected and uninfected (controls) individuals (see "Experimental Procedures" for details). The data presented are representative samples of randomly chosen individuals in each group. B, shown are Western blots of Grb2 and Grb3-3 from PBMCs of HIV-1-infected and uninfected individuals. Lane 1, NIH3T3 cells transfected with empty vector; lane 2, NIH3T3 cells transfected with a Grb3-3 expression construct; lane 3, PBMC lysate from seronegative individual; lanes 4–8, PBMC lysates from randomly chosen HIV-1-infected individuals. The Western blot was developed using an anti-Grb2 mAb (monoclonal antibody purchased from Upstate Biotechnology Inc., Lake Placid, NY) that recognizes Grb2 and Grb3-3 and was revealed by ECL. C, shown are the HIV-1 proteins expressed in Jurkat T-cells transfected with various viral protein expression vectors. Note that the difference in intensity among these bands is most probably due to a difference in the sources and/or the affinity of the antibodies used in the blots and should not be interpreted as a result of unequal transfection efficiency because transfection efficiency was routinely monitored by cotransfection with a β -galactosidase expression vector (Invitrogen). D, shown is Grb2 and Grb3-3 proteins detected in Jurkat T-cells transfected with various constructs. Jurkat T-cells were transfected with various constructs (5 μ g of DNA/5 \times 10⁶ cells) using LipofectAMINE. Seventy-two hours post-transfection, cells were harvested for isolation of cellular proteins, which were subsequently analyzed by Western blotting using mAb 3B5. Lane 1, cells transfected with pSV-Grb3-3 (positive control); lane 2, transfection with the empty vector (negative control); lanes 3–8, cells transfected with various expression vectors for HIV-1 viral proteins. The transfection efficiencies and expression levels of these individual viral expression constructs in transfected Jurkat T-cells were found to be quite similar (data not shown).

treatment of the transfected Jurkat T-cells with anti-CD3/anti-CD28 antibodies. A similar magnitude of NFAT/AP-1 activation by Grb3-3 (~7-fold) was also observed after treatment with PMA plus ionomycin, a condition that is generally believed to mimic TCR stimulation, but with maximal efficiency (24). These results suggest that Grb3-3 is capable of potentiating NFAT/AP-1 activation in Jurkat T-cells following TCR stimulation.

Because the luciferase reporter construct used in the above experiments of Fig. 3A consists of three tandem repeats of the composite NFAT/AP-1-binding site, we set out to determine which components were primarily involved. To this end, we performed the same experiments described above, but replaced the 3xNFAT/AP-1-LUC reporter with either the 3xAP-1-LUC or 3xNFAT-LUC reporter. As is shown Fig. 3B, no difference in activation of AP-1 was observed between Grb3-3 and the vector control upon stimulation with either anti-CD3/anti-CD28 anti-

bodies or PMA/ionomycin. In contrast, a 2–3-fold increase in activation of NFAT was detected with Grb3-3 when the 3xNFAT-LUC reporter was used (Fig. 3C). The magnitude of stimulation (2–3-fold) using 3xNFAT-LUC (Fig. 3C) was not as high as that using 3xNFAT/AP-1-LUC (~10-fold) (Fig. 3A) since the latter consists of composite binding elements for additional and cooperative binding between the NFAT and AP-1 transcription factors. In addition, we failed to observe any difference in stimulation of the nuclear factor κ B-driven reporter between Grb3-3 and the vector (data not shown). Interestingly, we did not observe significant effects of exogenous Grb2, through transfection of a Grb2 expression construct, on NFAT activity under similar experimental conditions (data not shown). Presumably, the endogenous Grb2 level is already so high that additional effects could not be detected under our experimental conditions. Collectively, these results suggest that Grb3-3 is capable of potentiating NFAT activity.

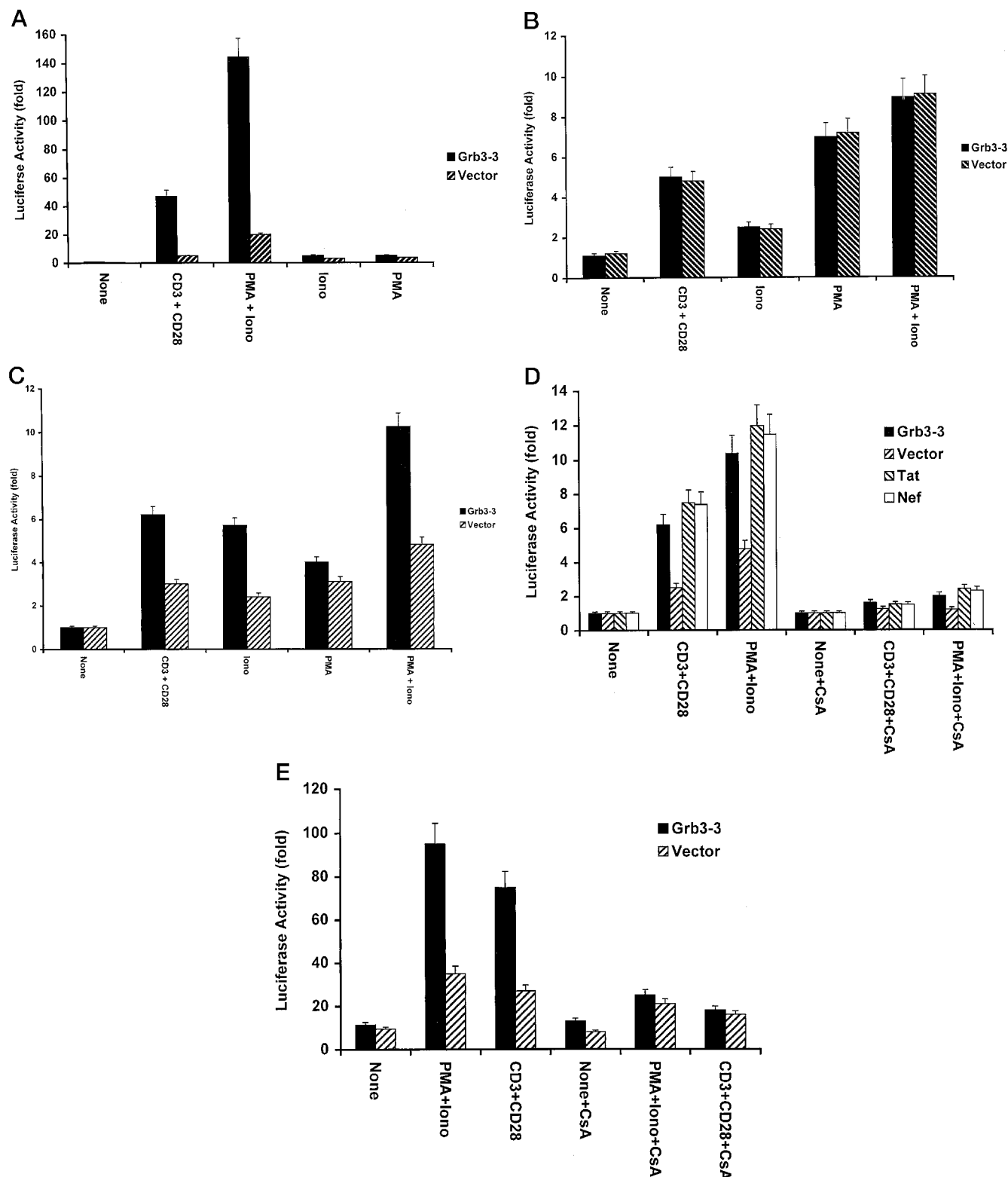


FIG. 3. Grb3-3 potentiates NFAT activation upon engagement of the TCR and CD28 co-receptor. *A*, effects of Grb3-3 on NFAT/AP-1 activation. Jurkat T-cells were transfected with 2 μ g of luciferase reporter (3xNFAT/AP-1-LUC) and 0.5 μ g of NFATc expression vector (48) in addition to either Grb3-3 or the empty vector (4 μ g). The total amount of DNA was also brought to 10 μ g using the empty vector. Cells were treated with anti-CD3/CD-28 antibodies, PMA, ionomycin (*Iono*), or a combination of PMA and ionomycin to facilitate dephosphorylation of NFAT (24). Luciferase activities were determined 72 h post-transfection (for details, see "Experimental Procedures"). Note that duplicate tests were usually performed simultaneously and that the experiments were repeated three times. Differences between each group falls between 5 and 10%. *Error bars* represent S.D. *B*, effects of Grb3-3 on AP-1 activation. Jurkat T-cells were transfected with the 3xAP-1-LUC plasmid together with either Grb3-3 or the empty vector. Seventy-two hours later, luciferase activities were analyzed. *C*, effects of Grb3-3 on NFAT activation. Jurkat T-cells were transfected with the 3xNFAT-LUC plasmid together with either Grb3-3 or the empty vector. Seventy-two hours later, luciferase activities were analyzed. *D*, effects of Tat and Nef on NFAT activation. Jurkat T-cells were transfected with the 3xNFAT-LUC plasmid together with Grb3-3, Tat, Nef, or the empty vector. In some cases, Csa (1 μ g/ml) was used at the same time that the stimuli (PMA, ionomycin, anti-CD3 antibody, or anti-CD28 antibody) were applied. *E*, effects of Grb3-3 on HIV-1 LTR promoter activity. Jurkat T-cells were transfected with the HIV LTR-driven luciferase construct together with either Grb3-3 or the empty vector. Treatment of the cells with stimuli and/or Csa and analysis of luciferase activity were as described above.

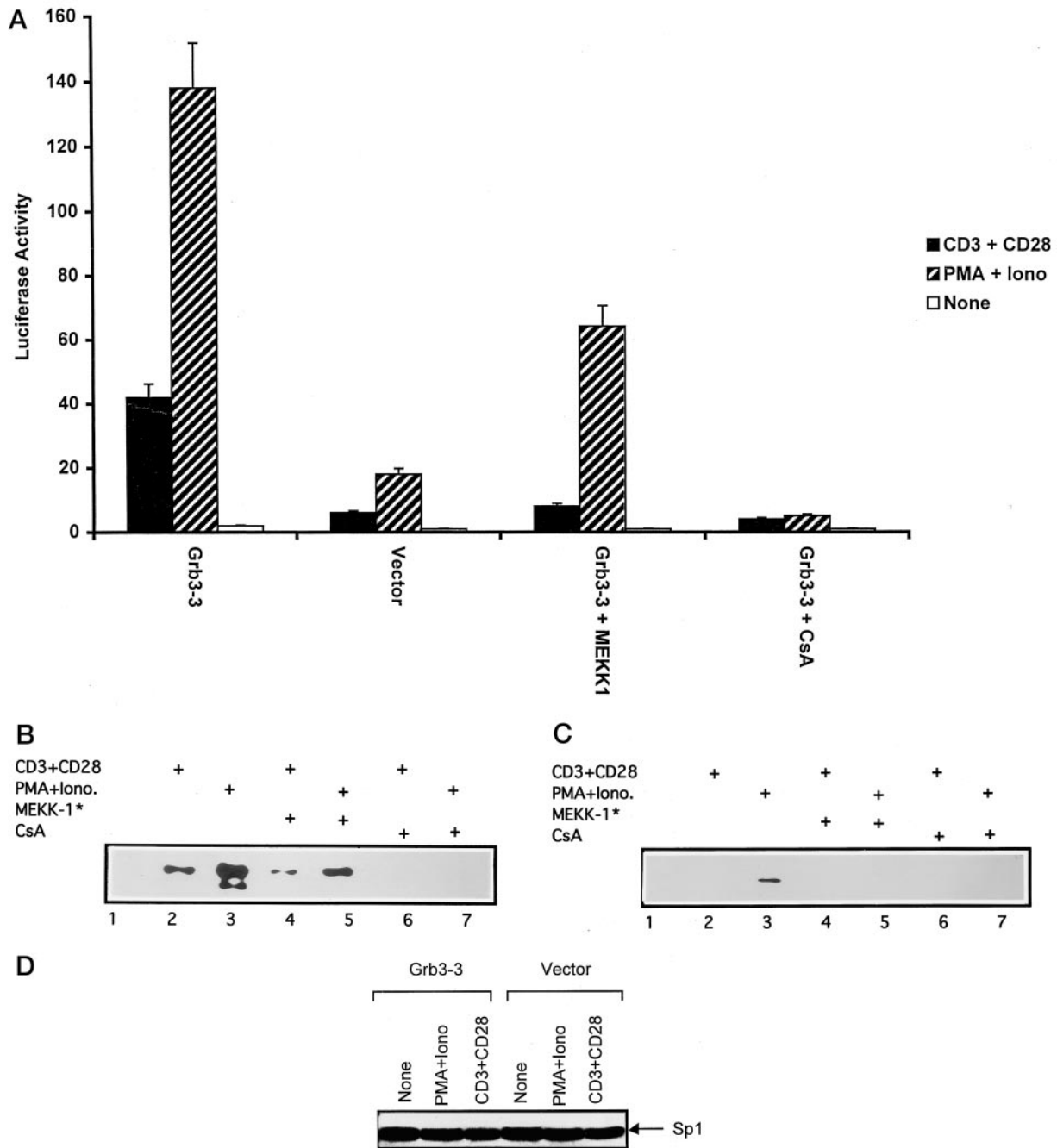


FIG. 4. Effects of Grb3-3 on NFAT nuclear localization. A, shown are the effects of MEKK1 and CsA on Grb3-3-induced NFAT activation. Experiments were performed as described in the legend to Fig. 3 with either cotransfection of constitutively activated MEKK1* (4 μ g) or treatment with CsA (1 μ g/ml). Values are means \pm S.D. B and C, shown is the detection of NFATc in the nuclear extracts of Jurkat cells transfected with either Grb3-3 or the empty vector control, respectively. Lane 1, no treatment; lane 2, treatment with anti-CD3/anti-CD28 antibodies; lane 3, treatment with PMA and ionomycin (Iono); lane 4, treatment with anti-CD3/anti-CD28 antibodies plus cotransfection with constitutively activated MEKK1*; lane 5, treatment with PMA/ionomycin plus cotransfection with constitutively activated MEKK1*; lane 6, treatment with anti-CD3/CD28 antibodies in addition to CsA; lane 7, treatment with PMA/ionomycin in addition to CsA. D, as an internal control, anti-Sp1 antibody was used to detect Sp1 in nuclear extracts under various conditions.

HIV-1 Tat and Nef Potentiation of NFAT Promoter Activity and Grb3-3 Potentiation of HIV-1 LTR Promoter Activity Are Both Sensitive to CsA—Since Tat and Nef can induce Grb3-3 expression and Grb3-3, in turn, can potentiate NFAT activities, we investigated whether Tat and Nef could potentiate NFAT activities. As is shown in Fig. 3D, both Tat and Nef caused an \sim 3-fold increase in luciferase activity. In addition, CsA, a drug that is known to oppose the function of calcineurin (15, 16), thereby blocking nuclear targeting of NFAT, substantially suppressed this activation of Tat, Nef, and Grb3-3 (Fig. 3D). These

results are in agreement with previous studies that Tat and Nef can activate several transcription factors including NFAT (38–45, 52–57).

NFATc has been reported to activate the HIV-1 LTR promoter (22). We next reasoned that Grb3-3 should be able to activate the HIV-1 LTR promoter because Grb3-3 was able to potentiate NFAT activity, as shown in Fig. 3 (A and C). To test this possibility, the HIV-1 LTR-driven luciferase reporter was cotransfected with the Grb3-3 expression vector into Jurkat T-cells. As expected, transfection of the Grb3-3 expression vec-

tor resulted in greater activation of the HIV-1 LTR promoter compared with the vector control, and CsA substantially suppressed potentiation of HIV-1 LTR activity by Grb3-3 (Fig. 3E). These results suggest that Grb3-3, up-regulated by HIV-1 infection, can potentiate NFAT activity and, as a result, increase HIV-1 LTR responsiveness following T-cell receptor/co-receptor stimulation.

Blocking Grb3-3-induced NFAT Nuclear Localization by Constitutively Activated MEKK1* and Cyclosporin A—In addition to the immunosuppressive drug CsA, cellular kinases such as MEKK1 have been implicated to oppose the function of calcineurin (15, 16), thereby blocking nuclear targeting of NFAT. Recently, Grb3-3 has also been demonstrated to bind MEKK1 *in vivo* (49). To extend our study, we analyzed the effects of MEKK1 on Grb3-3-potentiated NFAT nuclear localization. Fig. 4A shows that constitutively activated MEKK1* reduced the Grb3-3-potentiated NFAT activity that was seen following anti-CD3/anti-CD28 antibody and PMA/ionomycin stimulation by ~85 and 53%, respectively. As a control, CsA reduced the Grb3-3 potentiation of NFAT activity following anti-CD3/anti-CD28 antibody and PMA/ionomycin stimulation by ~93–97%.

The above observations were further strengthened by examination of NFAT proteins in nuclear extracts in immunoblot experiments using antibodies against NFATc following transfection of Jurkat T-cells with the Grb3-3 expression (Fig. 4B) or vector (Fig. 4C) plasmid. Clearly, NFAT was not detected in the nuclear extracts in the absence of any stimuli (Fig. 4B, lane 1). As expected, treatment of Grb3-3-transfected cells with anti-CD3/anti-CD28 antibodies resulted in nuclear targeting of NFATc (lane 2), whereas PMA and ionomycin treatment was found to maximally stimulate NFATc translocation into the nucleus (lane 3). However, additional treatment of these cells by cotransfection with constitutively activated MEKK1* (lanes 4 and 5) or CsA (lanes 6 and 7) was found to substantially suppress NFATc translocation into the nucleus. Fig. 4C shows the results of similar Western blot experiments, except that the control vector was used in place of the Grb3-3 expression plasmid. In these studies, nuclear NFAT was barely detectable in the same Western blots except in lane 3, where the strongest stimulation (PMA plus ionomycin) was applied; however, the NFATc protein band disappeared following additional treatment with either constitutively activated MEKK1* or CsA (lanes 5 and 7). It should be mentioned that the Western blot assay is not as sensitive as the luciferase reporter assay; therefore, the NFATc band in nuclear extracts following treatment with anti-CD3/anti-CD28 antibodies (Fig. 4C, lane 2) was undetectable, although an increase in luciferase activity could be detected (Fig. 4A). However, the results obtained in Fig. 4 (B and C) are consistent with those of Fig. 4A, *i.e.* Grb3-3 potentiated NFATc activity, and such effects were inhibited by either treatment with CsA or transfection with constitutively activated MEKK1*. As an internal control, transcription factor Sp1 was probed in nuclear extracts under similar conditions. As is shown in Fig. 4D, Grb3-3 exerted no detectable effect on the Sp1 level in the nucleus upon PMA/ionomycin or anti-CD3/anti-CD28 antibody stimulation. These results indicate that Grb3-3 can facilitate NFAT nuclear translocation upon TCR stimulation.

The mechanism(s) involving facilitation of NFAT nuclear translocation by Grb3-3 is currently unclear. CsA blocks NFAT activation primarily by inhibiting the phosphatase activity of calcineurin (15–20). However, the role of MEKK1 in inhibition of NFAT activation is less well understood. MEKK1 may act through intermediates such as SEK1 and JNKs in retaining NFAT in the cytoplasm, as JNKs have been shown to directly

phosphorylate NFAT (55). Alternatively, MEKK1 may coordinate with other kinases such as casein kinase I in masking the nuclear import signal of NFAT (46). Although Grb3-3 has been demonstrated to bind to MEKK1 (49), additional work will be needed to determine whether Grb3-3 exerts its effects through interaction with MEKK1 and/or to reveal the identities of other intermediates that might be involved in this process.

Grb3-3 has been reported to inhibit epidermal growth factor-induced transactivation of a Ras-responsive element, whereas Grb2 was found to overcome Grb3-3 inhibition in this pathway (31), suggesting that Grb3-3 may act as a suppressor over Grb2 in inhibiting the epidermal growth factor proliferative signals. However, the positive effects of Grb3-3 on NFAT potentiation in this report imply that Grb3-3 may not be restricted to work in the same pathway as Grb2 or to necessarily counteract the functional activity of Grb2 especially since Grb2 also plays a positive role in T-cell activation (32). Indeed, it is likely that signal pathways associated with the Ras-responsive element triggered by the epidermal growth factor (31) may not significantly contribute to NFAT activation because Grb3-3 works as a negative factor in this case.

In summary, our findings show that Grb3-3 is a cellular factor that can be up-regulated by HIV-1 and may act as a positive regulator for T-cell activation and HIV-1 replication through NFATc. Establishing the pathway(s) by which Tat and Nef induce expression of this Grb2 isoform and further clarifying its positive role in T-cell activation will aid in our understanding of how HIV-1 modulates expression of cellular signaling molecules to establish an intracellular environment that can favorably support its replication.

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