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Physical Interactions between Ets and NF-κB/NFAT Proteins Play an Important Role in Their Cooperative Activation of the Human Immunodeficiency Virus Enhancer in T Cells

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The transcriptional regulatory elements of many inducible T-cell genes contain adjacent or overlapping binding sites for the Ets and NF-κB/NFAT families of transcription factors. Similar arrays of functionally important NF-κB/NFAT and Ets binding sites are present in the transcriptional enhancers of human immunodeficiency viruses types 1 and 2 (HIV-1 and HIV-2), suggesting that this pattern of nuclear protein binding sites reflects an evolutionarily conserved mechanism for regulating inducible T-cell gene expression that has been co-opted during HIV evolution. Despite these findings, the molecular mechanisms by which Ets and NF-κB/NFAT proteins cooperatively regulate inducible T-cell gene expression remained unknown. In the studies described in this report, we demonstrated a physical interaction between multiple Ets and NF-κB/NFAT proteins both in vitro and in activated normal human T cells. This interaction is mediated by the Ets domain of Ets proteins and the C-terminal region of the Rel homology domains of NF-κB/NFAT proteins. In addition, the Ets–NF-κB/NFAT interaction requires the presence of DNA binding sites for both proteins, as it is abolished by the DNA intercalating agents propidium iodide and ethidium bromide and enhanced by the presence of synthetic oligonucleotides containing binding sites for Ets and NF-κB proteins. A dominant-negative mutant of NF-κB p50 that binds DNA but fails to interact with Ets proteins inhibits the synergistic activation of the HIV-1 and HIV-2 enhancers by NF-κB (p50 + p65) and Ets-1, suggesting that physical interaction between Ets and NF-κB proteins is required for the transcriptional activity of the HIV-1 and HIV-2 enhancers. Taken together, these findings suggest that evolutionarily conserved physical interactions between Ets and NF-κB/NFAT proteins are important in regulating the inducible expression of T-cell genes and viruses. These interactions represent a potential target for the development of novel immunosuppressive and antiviral therapies.

The lineage-specific and inducible expression of many eukaryotic genes is controlled by the assembly of multipartite complexes of transcription factors on promoter and enhancer elements containing complex arrays of sequence-specific nuclear protein binding sites. The assembly of these transcription factor complexes on DNA is regulated both by the sequences and the geometry of the nuclear protein binding sites and by physical interactions between the transcriptional regulatory proteins themselves. In many cases, viral pathogens have co-opted these cellular regulatory pathways by evolving transcriptional regulatory elements that resemble eukaryotic enhancers and that contain binding sites for multiple cellular transcription factors. For example, previous studies have demonstrated that the inducible expression of human immunodeficiency virus types 1 (HIV-1) and 2 (HIV-2) in T cells is regulated by viral enhancers that contain binding sites for multiple lymphoid-specific and ubiquitous host cell transcription factors, including NF-κB (14, 22, 77, 89), Ets (35, 38, 52, 86, 89), NFAT (15, 21, 25), and Sp1 (61, 89) proteins. Interestingly, adjacent and/or overlapping Ets and NF-κB/NFAT binding sites have also been identified in the regulatory elements of several inducible lymphoid genes, including those for interleukin-3 (IL-3) (18, 19), granulocyte-macrophage colony-stimulating factor (18, 66, 87, 101), IL-2 (15, 41, 42, 79, 80, 87), and IL-2 receptor alpha (44, 54, 95). Thus, this array of nuclear protein binding sites may reflect a conserved mechanism for regulating inducible lymphoid gene expression.

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Members of the NF-κB family, which include p50, p65, p105, p100, p52, c-Rel, and RelB, regulate gene expression in response to a wide variety of extracellular signals and are known to play important roles in the development and function of multiple mammalian cell lineages (57, 99). Preformed NF-κB proteins are sequestered in the cytoplasm in an inactive form by their association with members of the inhibitory IκB protein family (4, 94). In response to a variety of extracellular signals, IκB proteins are phosphorylated and degraded (26), thereby releasing transcriptionally active NF-κB proteins which translocate to the nucleus to regulate the expression of multiple target genes (1, 5, 6, 50). Recently, members of the NFAT family of transcription factors, which includes NFAT1/NFATp (55, 67), NFATc (55, 73), NFAT3/NFATX (37, 39, 65), and NFAT4 (39), have been identified as distantly related members of the NF-κB family. The DNA binding domains of NFAT and NF-κB proteins (termed the Rel homology domains [Rel HDs]) display low-level but significant sequence identity (41, 42, 73). Moreover, some NFAT proteins are sequestered in the cytoplasm of resting T cells (24, 59, 88, 107) and are translocated to the nucleus following T-cell activation (58, 83).

Several lines of evidence suggest that NF-κB/NFAT family members play an important role in regulating the inducible expression of both HIV-1 and HIV-2 in T cells. First, functionally important binding sites for NF-κB and NFAT proteins have been identified in the two enhancers (15, 21, 35, 71). Second, overexpression of NF-κB has been shown to transactivate reporter constructs driven by the HIV-1 and HIV-2 enhancers (29, 62, 71, 84). Finally, NF-κB activates transcription and induces chromatin rearrangement of the HIV-1 en-

hancer in vitro in combination with other transcription factors, including Sp1, LEF-1, and TFE-3 (77, 89).

Like the NF-κB/NFAT proteins, Ets transcription factors belong to a large multigene family with at least 17 mammalian members (2, 20, 60, 97, 105). All Ets proteins share an evolutionarily conserved 82-amino-acid (aa) winged-helix-loop-helix DNA binding domain that is structurally distinct from that of the NF-κB/NFAT proteins (7, 47, 53, 74, 76, 98). Like NF-κB/NFAT family members, Ets proteins have also been shown to play important roles in regulating the expression of multiple eukaryotic genes and in the development and function of multiple hematopoietic cell lineages (8, 10, 11, 68, 70, 75, 85, 93, 108). A large body of evidence suggests that Ets proteins also play an important role in regulating the inducible expression of HIV-1 and HIV-2 in T cells. A functionally important Ets binding site has been identified in the HIV-1 enhancer (38, 40, 86). Ets-1, Ets-2, and Fli-1 have all been shown to bind to this site in vitro, and these proteins can transcriptionally transactivate reporter constructs containing multiple copies of this site 5′ of a heterologous promoter (38, 40, 86). In addition, NF-κB has been shown to synergistically activate transcription of the HIV-1 enhancer in vitro when it is combined with purified Sp1 and Ets proteins (77, 89). Similarly, two functionally important Ets binding sites have been identified in the HIV-2 enhancer, and both Elf-1 and Ets-1 have been shown to bind to these sites in vitro (16, 32, 52, 63, 64).

Despite the evidence that NF-κB/NFAT and Ets proteins are each important regulators of the inducible expression of HIV-1 and HIV-2, it remained unclear if members of these two families of transcription factors function independently or cooperatively to regulate inducible HIV gene expression. Moreover, it was unclear if direct physical interactions between NF-κB/NFAT and Ets proteins play a role in regulating their transcriptional activity on the HIV enhancers. In the studies described in this report, we have (i) demonstrated physical interactions between multiple Ets proteins and NF-κB/NFAT family members both in vitro and in normal human T cells, (ii) mapped the interaction domains to the DNA binding domains of each of the proteins (the Ets domains of the Ets proteins and the Rel HDs of the NF-κB/NFAT proteins), and (iii) shown that physical interactions between specific Ets and NF-κB/NFAT proteins are required for the ability of the proteins to synergistically regulate the inducible expression of the HIV-1 and HIV-2 enhancers. Taken together, these findings are consistent with a model in which evolutionarily conserved physical interactions between Ets and NF-κB/NFAT family members play an important role in regulating the inducible expression of multiple viral and cellular genes in lymphoid cells.

MATERIALS AND METHODS

Plasmids. A full-length cDNA for NFATc was obtained by reverse transcription-PCR done with a commercially available kit (Pharmacia Biotechnology, Uppsala, Sweden) used according to the manufacturer's instructions, with mRNA isolated from Jurkat T cells following 4 h of stimulation with ionomycin and phorbol myristate acetate, as described previously (73), and the following synthetic nucleotides: 5′ primer GGG CTC GAG GAT CCC ATG CCA AGC ACC AGC TTT C and 3′ primer GGG TCT AGA GGA TCC TTA GAA AAA GCA CCC CAC. Truncations of NFATc were made using PCR with the following primers: for $NFATc_{1–417}$, 5′ primer CCG CTC GAG CGG ATC CCC ATG CCA AGC ACC AGC TTT CCA GTC and 3′ primer TGC TCT AGA GGA TCC CTA CCA GTC CAG GGC GGG CAG GGT CGG; for $NFATc_{418–716}$, 5′ primer CCG CTC GAG CGG ATC CCC CAG CTG CCG TCC CAC TCA GGC CCG and 3′ primer TGC TCT AGA GGA TCC CTA CTC ATG TTC ACG GCT TAC GGT TAG.

Full-length and truncated NFATp, p50, p65, c-Rel, Elf-1 and Ets-1 cDNAs were prepared by PCR, using as templates the previously described plasmids pBS NFATp (67), pRSV p50 (49), pRSV p65 (81, 82), pRSV c-Rel (13, 22), pcDNA3 Ets-1 (3), pcDNA1-neoElf-1 (52) and the following oligonucleotide primers: for NFATp, 5′ primer CTA GTC TAG AGG CGG CTC CTC TGC CAG CTT CAT TTC and 3′ primer CTA GTC TAG ATC AGA TCC AGC CTG GGC TAG CAA C; for $NFATp_{1–520}$, 5′ primer CTA GTC TAG AGG CGG CTC CTC TGC CAG CTT CAT TTC and 3′ primer CTA GTC TAG ACT AAG ATG GTT CAT ACT CAT CGC TGG G; for $NFATp_{521–890}$, 5′ primer CTA GTC TAG ATT GAT CTG CAG CCC CGC CCA TGG AG and 3′ primer CTA GTC TAG ATC AGA TCC AGC CTG GGC TAG CAA C; for $p50_{36–367}$, 5′ primer GGG CCG CTC GAG CTG CCA ACA GCA GAT GGC and 3′ primer ATC GGG CCC CTA GAG CTT CTG ACG TTT CCT C; for p65, 5′ primer G CCG CTC GAG ATG GAC GAA CTG TTC CCC CTC and 3′ primer G TGC TCT AGA TTA GGA GCT GAT CTG ACT CAG CAG; for $p65_{11–304}$, 5′ primer GGG CCG CTC GAG GAG CCA GCC CAG GCC TCT G and 3′ primer GTG CTC TAG ACT ATG TCC TTT TAC GTT TCT CCT CAA TC; for c-$Rel_{1–295}$, 5′ primer GGG CCG CTC GAG ATG GCC TC GGT GCG TAT AAC and 3′ primer C TAG TCT AGA CTA TGT CTT TTG TTT CTT TGC; for Elf-1, 5′ primer GGG CTC GAG GAT CCC ATG GCT GCT GTT GTC CAA and 3′ primer CCC GGG TCT AGA TAA CTA AAA AGA GTT GGG TTC CAG C; for Elf-$1_{90–619}$, 5′ primer GGG CTC GAG GAT CCC AAA CCA GGG AAT TCT AAA GC and 3′ primer CCC GGG TCT AGA TAA CTA AAA AGA GTT GGG TTC CAG C; for Elf-$1_{196–295}$, 5′ primer GGG CTC GAG GAT CCC GTG AAG AAG AAA AAC AAA GAT GG and 3′ primer GGG TCT AGA GGA TCC CTA ATC TTT TGG CAT TTC TTT AAA C; for Ets-$1_{331–418}$, 5′ primer GGG CTC GAG GAT CCC GGC AGT GGA CCA ATC CAG CTA TGG and 3′ primer GGG TCT AGA GGA TCC CTA CAG GTC ACA CAC AAA GCG GTA C.

Full-length NFATc, NFATc, $NFATc_{418–716}$, $p50_{36–367}$, full-length p65, $p65_{11–304}$, c-$Rel_{1–295}$, full-length Elf-1, Elf-$1_{1–417}$, Elf-$1_{290–619}$, Elf-$1_{196–295}$, Elf-$1_{290–619}$, and Ets-$1_{331–410}$ cDNAs were cloned into the *Xho*I and *Xba*I sites of the pcDNA3HA plasmid, which contains an HA epitope tag (encoding amino acids M G Y P Y D V P D Y A S M) in the *Eco*RV and *Xho*I sites of the pcDNA3-Neo plasmid (Invitrogen, San Diego, Calif.). The full-length and truncated cDNAs of NFATp were cloned into the *Xba*I site of pcDNA3HA. The full-length cDNA for p50 was cloned into the *Xho*I and *Apa*I sites of pcDNA3; $p50_{36–367}$ was cloned into the *Xho*I-*Apa*I sites of pcDNA3HA. pcDNA3 Ets-1 (3) and pcDNA3 PU.1 (33) have been described previously.

The chimeras of p50 and p65 were constructed by the overlap extension method of PCR, using the following sets of primers: for p50/p65 chimera 1, 5′ primer 1 GGG CCG CTC GAG CTG CCA ACA GCA GAT GGC, 3′ primer 1 GCC TGA TGG GTC CCG CAC TGT AGC TGT AAA CAT GAG CCG CAC, 5′ primer 2 GTG CGG CTC ATG TTT ACA GCT ACA GTG CGG GAC CCA TCA GGC and 3′ primer 2 GTG CTC TAG ACT ATG TCC TTT TAC GTT TCT CCT CAA TC; for p65/p50 chimera 3, 5′ primer 1 GGG CCG CTC GAG GAG CCA GCC CAG GCC TCT G, 3′ primer 1 CAG TGC TAT CCG GAA GAA ACA CCT GGA AGC AGA GCC GCA C, 5′ primer 2 GTG CGG CTC TGC TTC CAG GTG TTT CTT CCG GAT AGC ACT G, and 3′ primer 2 ATC GGG CCC CTA GAG CTT CTG ACG TTT CCT C; for p50/p65 chimera 5, 5′ primer 1 GGG CCG CTC GAG CTG CCA ACA GCA GAT GGC, 3′ primer 1 CTC ACT GAG CTC CCG GTC GGA AGG CCT CCG AAG CTG GAC AAA CAC, 5′ primer 2 GTG TTT GTC CAG CTT CGG AGG CCT TCC GAC CGG GAG CTC AGT GAG, and 3′ primer 2 GTG CTC TAG ACT ATG TCC TTT TAC GTT TCT CCT CAA TC; for p65/p50 chimera 6, 5′ primer 1 GGG CCG CTC GAG GAG CCA GCC CAG GCC TCT G, 3′ primer 1 CCT CCG AAG CTG GAC AAA CAC CTC ACT GAG CTC CCG GTC GGA AGG, 5′ primer 2 CCT TCC GAC CGG GAG CTC AGT GAG GTG TTT GTC CAG CTT CGG AGG, and 3′ primer 2 ATC GGG CCC CTA GAG CTT CTG ACG TTT CCT C; for p50 rel/p65 chimera, 5′ primer 1 CCG CTC GAG ATG GAC GAA CTG TTC CCC CTC ATC TTC CCG GCA CTG CCA ACA GCA GAT GGC CC, 3′ primer 1 CAT GAT GCT CTT GAA GGT CTC ATA GAG CTT CTG ACG TTT CCT C, 5′ primer 2 G AGG AAA CGT CAG AAG CTC TAT GAG ACC TTC AAG AGC ATC ATG, and 3′ primer 2 GTG CTC TAG ATT AGG AGC TGA TCT GAC TCA GCA G. The products of the second PCR reactions for p50/p65 chimera 1, p50/p65 chimera 5, and p50 rel/p65 chimera were digested with *Xho*I and *Xba*I and cloned into the *Xho*I-*Xba*I sites of pcDNA3HA. The products of the second PCR reactions for p65/p50 chimera 3 and p65/p50 chimera 6 were digested with *Xho*I and *Apa*I and cloned into the *Xho*I-*Apa*I sites of pcDNA3HA.

The pGEX-Elf-1 plasmid encoding a glutathione *S*-transferase (GST)–Elf-1 fusion protein was described previously (103). The pGEX-NFATc plasmid contains the full-length NFATc cDNA (described above) cloned into the *Bam*HI site of the pGEX plasmid (Pharmacia Biotech).

The HIV-1 Luc plasmid contains the *Xho*I-*Hin*dIII fragment from HIV-1 CAT (71), containing the HIV-1 long terminal repeat (LTR), cloned into the *Xho*I and *Hin*dIII sites of pGL2 basic vector (Promega Corp., Madison, Wis.). The pBS wild-type (wt) HIV-2, pBS HIV-2 mκB, and pBS HIV-2 mEts constructs were prepared by PCR using as templates the previously described HIV-2 CAT, HIV-2 mκBCAT, and HIV-2 mPUB2 CAT plasmids (52) with the following synthetic oligonucleotide primers: 5′ primer CGC GGA TCC AAT ACC ATT TAG TTA AAG and 3′ primer CGC GGA TCC GGT TAC AGC CCC TTC TGG. The products of these PCR reactions were digested with *Bam*HI and cloned into the *Bam*HI sites of pBS KS^- (Stratagene Corp., La Jolla, Calif.). HIV-2wtLuc, HIV-2mκBLuc, and HIV-2mEtsLuc were constructed by cloning the HIV-2 promoter/enhancers from HIV-2 CAT, HIV-2 mκBCAT, and HIV-2

mPUB2 CAT plasmids (52) into the *Stu*I-*Hin*dIII sites of the pGL2 basic vector. The identities of all constructs made by using PCR were confirmed by dideoxy DNA sequence analysis.

Antibodies. The α-CREM, α-Myc, α-Myb, and α-p50 polyclonal antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, Calif.). The α-NFAT antibody was purchased from Upstate Biotechnology Inc. (Lake Placid, N.Y.). The α-Elf-1 polyclonal antibody and the 6G7 (101) α-Elf-1 monoclonal antibody (MAb) were described previously (3).

EMSAs. A double-stranded oligonucleotide derived from the HIV-2 LTR NF-κB site (CAGCTGAGACTGCAGGGACTTTCCAGAAGGGGCTG) (52) and containing overhanging *Bam*HI-*Bgl*II ends was labeled with ^{32}P nucleotides by using the Klenow fragment of DNA polymerase I and used as a probe in electrophoretic mobility shift assays as described previously (101). For electrophoretic mobility shift assays (EMSAs) using either wild-type or mutant versions of the −186 to −83 region from the HIV-2 LTR as a probe, the *Bam*HI fragments from either pBS HIV-2 wt oligo, pBS HIV-2 mκB oligo, or pBS HIV-2 mEts oligo were excised, gel purified, and labeled with ^{32}P nucleotides by using the Klenow fragment of DNA polymerase I as described above. For supershift experiments, 1 μl of the α-CREM, α-p50, or α-Elf-1 polyclonal antibody was added to the reactions.

In vitro transcription and translation reactions. In vitro transcription and translation reactions were carried out in a rabbit reticulocyte lysate system, using a commercially available kit (Promega Corp., Madison, Wis.) according to the instructions of the manufacturer, as described previously (104).

GST fusion proteins and binding assays. Glutathione-Sepahrose beads (Pharmacia) were washed three times in NETN (20 mM Tris [pH 8.0], 100 mM NaCl, 1 mM EDTA, and 0.5% Nonidet P-40) plus 0.5% powdered milk. Preparations of ^{35}S-labeled in vitro-translated NF-κB/NFAT and Ets proteins were precleared by incubation with 30 μl of glutathione-Sepharose beads (Pharmacia) in 500 μl of NETN for 1 h at 4°C. Overnight cultures of *Escherichia coli* DH5α containing either the pGEX-3X (Pharmacia), pGEX-Elf-1, or pGEX-NFATc plasmid were diluted 1:10 in Luria broth and grown for 1 h with shaking at 37°C. Isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 0.1 mM, and cultures were incubated at 37°C for 4 h with shaking. Bacteria were pelleted and resuspended in NETN. After incubation at 4°C for 30 min, the cells were sonicated three times for 15 s each time on ice. Equivalent amounts of GST fusion proteins (as determined by Coomassie blue staining) were bound to 30 μl of glutathione-Sepharose beads by incubation in a total volume of 1 ml of NETN for 1 h at 4°C. The beads were washed three times with NETN and mixed with equivalent molar amounts (as determined by phosphorimager analysis) of precleared, ^{35}S-labeled, in vitro-translated proteins at 4°C for 1 h. The beads were incubated with NETN, and the proteins were eluted by boiling for 3 min in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer (50 mM Tris [pH 6.8], 30% glycerol, 0.4% SDS, 0.1% bromophenol blue) containing 10% β-mercaptoethanol. For the ethidium bromide (EtBr) experiments, beads were incubated with NETN containing either 10 or 100 μg of EtBr per ml. For the propidium iodide (PI) experiments, beads were incubated with NETN containing either 7.5 or 15 μg of PI per ml. The proteins were separated by SDS-PAGE.

Isolation of human peripheral blood T cells. Human peripheral blood lymphocytes were isolated from buffy coat cells obtained by leukapheresis. T cells were isolated with a commercially available column according to the manufacturer's instructions (Research and Development Systems, Boulder, Colo.). Cells purified by this method are >98% T cells as determined by fluorescence-activated cell sorting analysis with an α-CD3 MAb. Purified T cells were cultured at a concentration of 2×10^6/ml in RPMI 1640 medium (GIBCO, Grand Island, N.Y.) supplemented with 10% heat-inactivated fetal calf serum (GIBCO), 2 mM glutamine, 100 U of penicillin G per ml, and 15 mM HEPES (pH 7.4) (GIBCO). T cells were activated by treatment for 8 h with phorbal myristate acetate (10 ng/ml) plus ionomycin (0.4 μg/ml).

Immunoprecipitations. Activated human T cells (0.5×10^8 to 1×10^8) cells were washed with ice-cold phosphate-buffered saline and lysed by incubation for 15 min at 4°C in 1 ml of NETN plus protease inhibitors (0.1 mM aprotinin, 1 mM leupeptin, and 0.1 mM phenylmethylsulfonyl fluoride). Cell extracts were cleared by centrifugation in a microcentrifuge at 16,000 × *g* for 15 min at 4°C. Cell extract (1 ml) was mixed with 10 μl of α-CREM, α-Myc, α-Myb, and α-p50 or α-NFAT and incubated for 1 h at 4°C. Protein A-Sepharose (50 μl) was added to each immunoprecipitation reaction mixture and incubated for 1 h at 4°C. The beads were washed five times with NETN, and the proteins were eluted by boiling in SDS-PAGE loading buffer. The proteins were resolved by electrophoresis in SDS–8% polyacrylamide gels. Immunoblots were probed with a 1:500 dilution of the α-Elf-1 MAb 6G7 in conjunction with a 1:3,000 dilution of a commercially available horseradish peroxidase-coupled goat antibody to mouse immunoglobulin (GIBCO). Immunoblots were developed by using a commercially available kit (Amersham, Arlington Heights, Ill.) as described previously (103).

Transfections and luciferase assays. COS cells were grown in Dulbecco's modified Eagle's medium with 4.5 g of glucose per liter supplemented with 10% fetal bovine serum and penicillin-streptomycin (GIBCO). Exponentially growing cultures containing 6×10^5 COS cells were transiently transfected with 1 μg of the HIV-1 Luc reporter plasmid and various amounts of Ets-1 and NF-κB eukaryotic expression plasmids by using Lipofectamine reagent (GIBCO) according to the manufacturer's instructions. Each transfection mixture contained a total of 21 μg of DNA including 1 μg of the RSV–β-galactosidase reference plasmid and 57 μl of Lipofectamine. Cell lysates were prepared 48 h after transfection, normalized for protein content by using a commercially available kit (Bio-Rad, Hercules, Calif.), and assayed for luciferase and β-galactosidase activities as described previously (101).

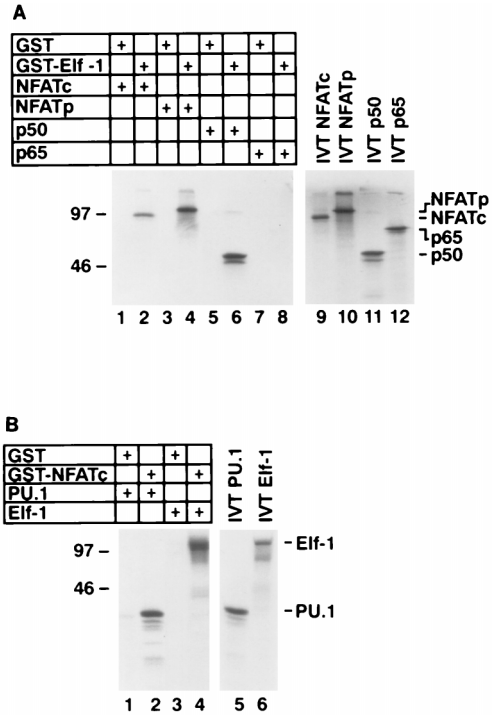

FIG. 1. (A) Physical association between Elf-1 and NFATc, NFATp, and p50. Equivalent molar amounts of ^{35}S-labeled in vitro-translated (IVT) NFATc, NFATp, p50, and p65 proteins were mixed with 1 μg of bacterially expressed GST–Elf-1 or an equivalent amount of the GST control protein. The protein complexes were denatured and separated by SDS-PAGE prior to autoradiography. (B) Association of Elf-1 and PU.1 with NFATc. Equivalent molar amounts of ^{35}S-labeled in vitro-translated Elf-1 and PU.1 were mixed with 1 μg of bacterially expressed GST-NFAT or an equivalent amount of the GST control protein. ^{35}S-labeled in vitro-translated NF-κB/NFAT proteins were run directly in parallel on the same gel. The resulting complexes were separated by SDS-PAGE. Size markers (in kilodaltons) are shown to the left of each autoradiogram.

RESULTS

A physical interaction between Ets and NF-κB/NFAT transcription factors. Previous studies have demonstrated functionally important and adjacent (and/or overlapping) binding sites for Ets and NF-κB/NFAT transcription factors in multiple inducible lymphoid genes, including those for human IL-2 (15, 42, 87), IL-3 (18, 19, 28), IL-2 receptor alpha (44, 54, 95), and granulocyte-macrophage colony stimulating factor (18, 66, 87, 101), as well as in viral transcriptional regulatory elements including the HIV-1 and HIV-2 enhancers (38, 52, 71, 77, 89). To determine whether Ets and NF-κB/NFAT proteins can associate in vitro, full-length recombinant bacterial GST–Elf-1 fusion protein was incubated with equivalent molar amounts of ^{35}S-labeled in vitro-translated NFATc, NFATp, p50, or p65 proteins (Fig. 1A). NFATc, NFATp, and p50 each associated efficiently with GST–Elf-1 (Fig. 1A, lanes 2, 4, and 6). The associations between GST–Elf-1 and NFATc, NFATp, and p50 were specific because none of these proteins associated with GST alone (Fig. 1A, lanes 1, 3, and 5) and because GST–Elf-1 did not associate with the structurally related NF-κB family member p65 (Fig. 1A, lane 8). In reciprocal experiments, full-

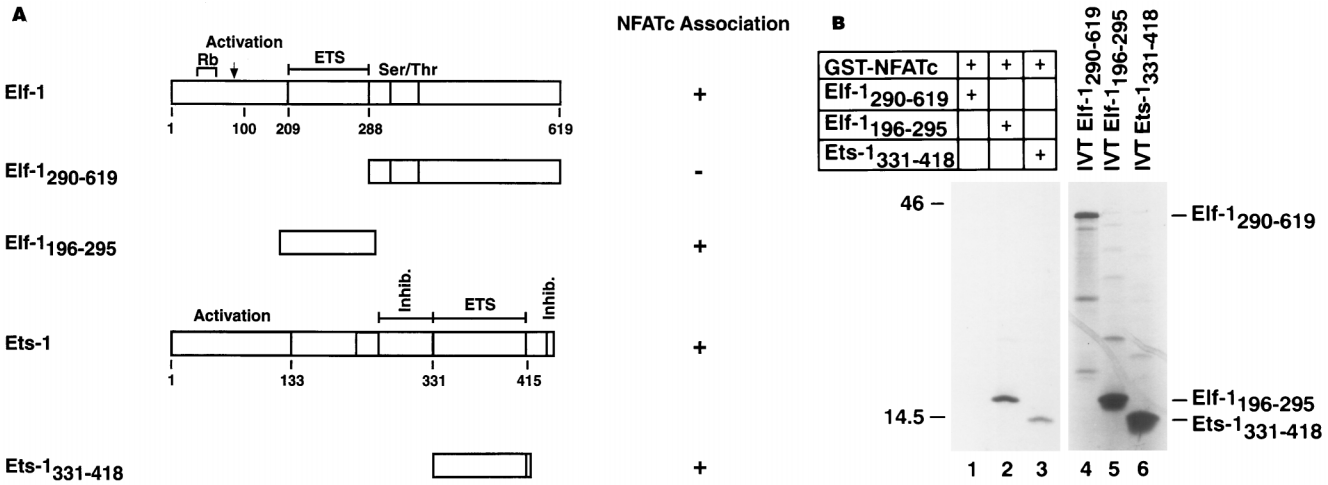

FIG. 2. (A) Schematic illustration of full-length and truncated Elf-1 and Ets-1 proteins. The positions of the Ets domains of Elf-1 and Ets-1 (ETS) (96), the transcriptional transactivation domains of Ets-1 and Elf-1 (Activation) (103), the Rb binding domain of Elf-1 (Rb) (103), the serine- and threonine-rich region of Elf-1 (Ser/Thr) (96), and the inhibitory domains of Ets-1 (Inhib.) (30, 31, 45, 56, 78, 106) are shown. Amino acid numbers are shown below the schematic illustrations of the full-length Elf-1 and Ets-1 proteins. Binding (+) or lack of binding (−) to GST-NFATc is indicated under NFATc Association. (B) NFATc interacts with the Ets domains of Elf-1 and Ets-1. ^{35}S-labeled in vitro-translated Ets proteins shown in panel A were mixed with 1 μg of GST-NFATc and analyzed by SDS-PAGE. ^{35}S-labeled in vitro-translated (IVT) Ets proteins were run directly in parallel on the same gel (right panel). Size markers (in kilodaltons) are shown to the left of the autoradiogram.

length recombinant bacterial GST-NFATc fusion protein was incubated with equivalent molar amounts of ^{35}S-labeled in vitro-translated Ets-related proteins Elf-1 and PU.1 (Fig. 1B). Both Elf-1 and PU.1 associated specifically and efficiently with GST-NFATc but not with GST alone. Taken together, these experiments demonstrated that the distantly related Ets proteins Elf-1 and PU.1 are capable of associating with several related but evolutionarily divergent NF-κB/NFAT proteins, including NFATc, NFATp, and p50. In contrast, Elf-1 does not associate with p65 in vitro.

The Ets domain is necessary and sufficient for the association of Ets and NF-κB/NFAT proteins. The 82-aa Ets domain is highly conserved between all Ets family members (47, 105). In this context, our finding that the distantly related Ets family members Elf-1 and PU.1 both associated efficiently with NFATc suggested that the association between Ets and NF-κB/NFAT proteins is mediated by the Ets domain of the Ets proteins. To test this hypothesis directly, GST-NFATc was mixed with different deletion mutants of ^{35}S-labeled in vitro-translated Ets proteins (Fig. 2B). The isolated Ets domain of Elf-1 (Elf-$1_{196–295}$) and the Ets domain of Ets-1 (Ets-$1_{331–418}$) each efficiently associated with GST-NFATc (Fig. 2B, lanes 2 and 3). In contrast, a deletion mutant of Elf-1 lacking the Ets domain (Elf-$1_{290–619}$) failed to associate with NFATc (Fig. 2B, lane 1). Taken together, these experiments demonstrated that the Ets domain is necessary and sufficient for the interaction of Ets transcription factors with NF-κB/NFAT proteins.

The Rel HDs of specific NF-κB/NFAT proteins are sufficient to mediate their interactions with Ets proteins. Having established that the Ets domains of Ets transcription factors are required for their interactions with NF-κB/NFAT, we attempted to map the region(s) of the NF-κB/NFAT proteins that is required for their interactions with Ets proteins. ^{35}S-labeled in vitro-translated deletion mutants of the NF-κB/NFAT proteins (Fig. 3A) were analyzed for their abilities to associate with GST–Elf-1. Full-length NFATc and NFATp both associated efficiently with GST–Elf-1 (Fig. 3B, lanes 1 and 4), as did deletion mutants containing only the Rel HDs of NFATc (NFATc$_{418–716}$), NFATp (NFATp$_{1–520}$), p50 (p50$_{36–367}$), and c-Rel (c-Rel$_{1–295}$) (Fig. 3B, lanes 3, 5, 7, and 8). In contrast, deletion mutants lacking the Rel HD of NFATc (NFATc$_{1–417}$) or NFATp (NFATp$_{521–890}$) failed to associate with GST–Elf-1 (Fig. 3B, lanes 2 and 6). In reciprocal experiments using different mutant GST–NF-κB/NFAT proteins (including NFATc, NFATp, and p50) and several different Ets proteins (including Elf-1, Ets-1, and PU.1), we found that the Rel HDs were uniformly required for the interaction between each of the NF-κB/NFAT and Ets proteins (data not shown). Taken together, these experiments demonstrated that the Rel HDs of NF-κB/NFAT proteins are both necessary and sufficient for their association with Ets family members.

Fine mapping of the Ets interaction domain of p50. The p65 protein contains a Rel HD that is more related to the Rel HD of p50 than to the Rel HDs of the NFAT proteins (73). Therefore, it was somewhat surprising that the full-length p65 protein, unlike the p50, NFATc, and c-Rel proteins, failed to bind to GST–Elf-1 (Fig. 1A). There were at least two possible explanations for this finding. First, it is possible that the p65 protein contains a region(s) outside of the Rel HD which inhibits the interaction between Elf-1 and p65. Alternatively, the Rel HD of p65 may lack an Ets binding motif. To distinguish between these possibilities, GST–Elf-1 was incubated with a peptide encompassing only the Rel HD of p65 (p65$_{11–304}$). Like wild-type p65, p65$_{11–304}$ failed to associate with GST–Elf-1 (Fig. 3B, lane 9). Thus, p65 does not contain a region outside of the Rel HD that inhibits its interaction with Ets proteins but, instead, lacks an Ets binding domain.

To map more finely the region of NF-κB/NFAT that is required for its interaction with Ets proteins, a series of chimeric proteins containing regions from p50 and p65 were constructed (Fig. 4A). ^{35}S-labeled in vitro-translated proteins produced from these mutants were assayed for their abilities to associate with GST–Elf-1 (Fig. 4B). Whereas chimeras containing the C-terminal 31 aa from the Rel HD of p50 (p50$_{36–367}$, p65/p50 chimera 3, and p65/p50 chimera 6) each associated efficiently with GST–Elf-1, mutant proteins lacking these 31 aa but containing instead the homologous 31-aa region from the Rel HD of p65 (p65$_{11–304}$, p50/p65 chimera 1, and p50/p65 chimera 5) failed to associate with GST–Elf-1. The abilities of these proteins to interact with Elf-1 were independent of their

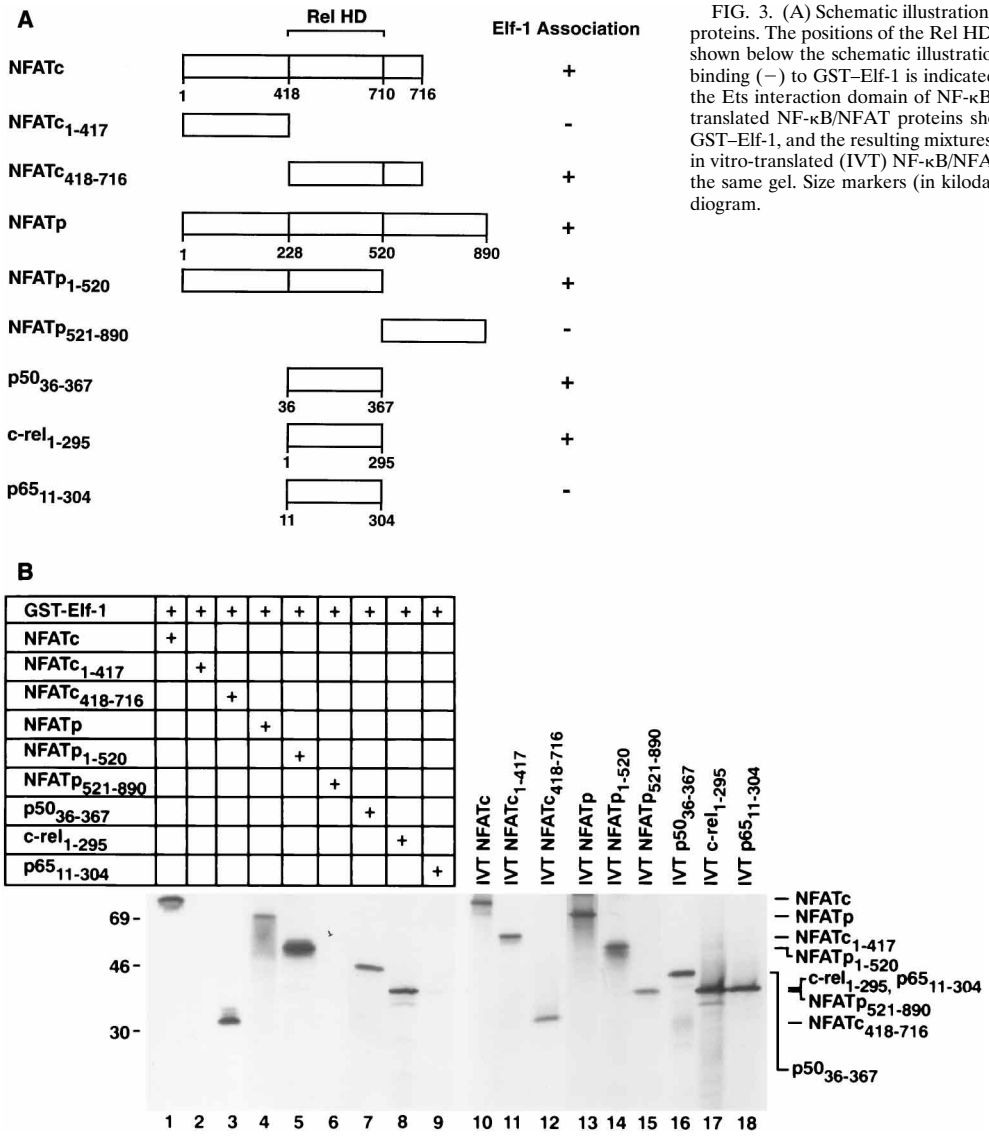

FIG. 3. (A) Schematic illustration of full-length and truncated NF-κB/NFAT proteins. The positions of the Rel HDs (73) are shown. Amino acid numbers are shown below the schematic illustrations of the proteins. Binding (+) or lack of binding (−) to GST–Elf-1 is indicated under Elf-1 Association. (B) Mapping of the Ets interaction domain of NF-κB/NFAT proteins. The ^{35}S-labeled in vitro-translated NF-κB/NFAT proteins shown in panel A were mixed with 1 μg of GST–Elf-1, and the resulting mixtures were analyzed by SDS-PAGE. ^{35}S-labeled in vitro-translated (IVT) NF-κB/NFAT proteins were run directly in parallel on the same gel. Size markers (in kilodaltons) are shown to the left of the autoradiogram.

abilities to bind DNA, since all of the mutant and chimeric proteins bound efficiently to the NF-κB site from the HIV-2 enhancer in EMSAs (Fig. 4B). Quantitative analyses of the protein-protein binding with a phosphorimager revealed that mutant proteins containing the C-terminal 31 aa from the Rel HD of p65 bound to GST–Elf-1 at least 30-fold less efficiently than proteins containing the homologous 31-aa region from the p50 Rel HD. Taken together, these experiments mapped the Ets binding site of p50 to aa 336 to 367 of the Rel HD.

Elf-1 associates with NF-κB/NFAT in activated normal human T cells. To demonstrate a physical interaction between Ets and NF-κB/NFAT in vivo, normal human T cells were activated for 8 h with phorbal myristate acetate plus ionomycin, and cell extracts were immunoprecipitated with antibodies specific for different NF-κB/NFAT proteins. These immunoprecipitates were subsequently denatured and subjected to immunoblot analysis with an α-Elf-1 MAb (Fig. 5). Elf-1 was coimmunoprecipitated with NFAT (Fig. 5, lane 4) and p50 (Fig. 5, lane 6). These coimmunoprecipitations represented specific Elf-1–NF-κB/NFAT interactions, because Elf-1 failed to coimmunoprecipitate in parallel experiments that used the same cell extracts and control antibodies directed against the CREM, c-Myc, and c-Myb proteins (Fig. 5, lanes 2, 3, and 5). Reciprocal experiments demonstrated that NFAT could be coimmunoprecipitated from activated human T-cell extracts by using a MAb directed against Elf-1 (data not shown). Taken together, these experiments demonstrated a physical interaction between the Ets-related protein Elf-1 and NF-κB/NFAT proteins in activated normal human T cells.

The Ets–NF-κB/NFAT interaction requires DNA binding sites for both proteins. To determine whether the Elf-1–NF-κB/NFAT interactions required DNA binding sites for these proteins, the in vitro binding experiments were repeated in the presence of EtBr, which has been shown to disrupt DNA-protein interactions (51). Both the Elf-1–p50 and the Elf-1–NFATc interactions were significantly reduced in the presence of EtBr (Fig. 6A, lanes 5 and 6; Fig. 6B, lane 4). The abrogation of the Elf-1–NF-κB and Elf-1–NFATc complexes by EtBr was specific for the NF-κB/NFAT–Ets interactions because similar concentrations of EtBr did not affect the previously described physical interaction between the Elf-1 and c-Jun proteins, which does not require DNA (3) (Fig. 6A, lanes 2 and

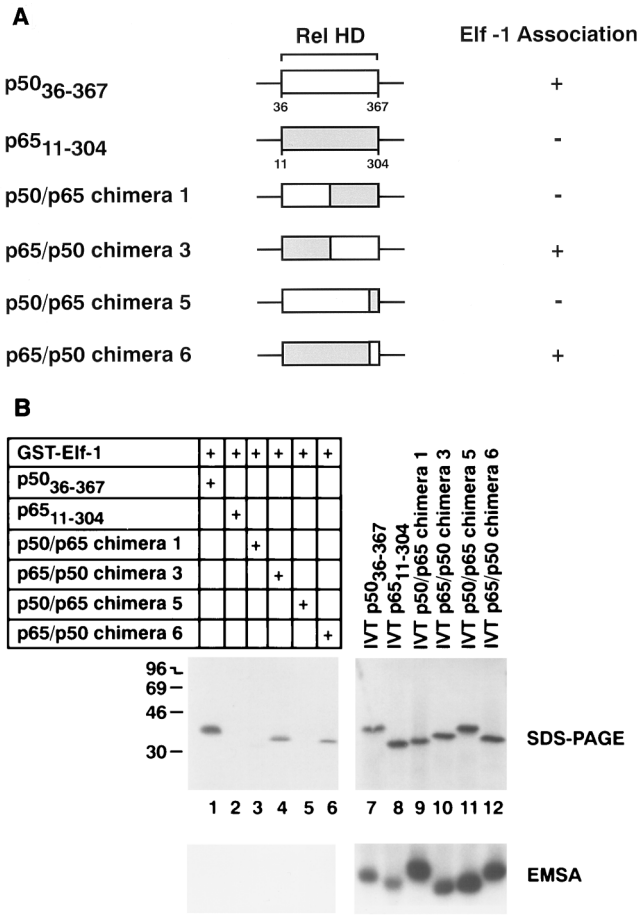

FIG. 4. (A) Schematic illustration of the chimeric NF-κB p50 and p65 proteins. The Rel HDs are shown (73). Regions containing amino acids from p50 are indicated in white, while regions containing amino acids from p65 are shaded. Binding (+) or lack of binding (−) of these chimeric proteins to GST–Elf-1 is indicated under Elf-1 Association. (B) Fine mapping of the Ets interaction domain of p50. The ^{35}S-labeled in vitro-translated proteins shown in panel A were incubated with 1 μg of GST–Elf-1, and the resulting mixtures were analyzed by SDS-PAGE. Size markers (in kilodaltons) are shown to the left of the autoradiogram. The in vitro-translated (IVT) chimeric proteins were also used in EMSAs with a ^{32}P-labeled NF-κB oligonucleotide from the HIV-2 enhancer (EMSA). Note that all of the chimeric proteins retain DNA binding activity.

3; Fig. 6B, lanes 2 and 3). To exclude the possibility that the observed disruption of the Elf-1–NF-κB/NFAT interaction by EtBr was due to a nonspecific effect of EtBr on the NF-κB/NFAT or Ets protein, a second set of binding experiments was performed in the presence of the DNA intercalating agent PI, which has also been shown to specifically disrupt DNA-protein interactions (51). Consistent with our results with EtBr, the Elf-1–p50 interaction was eliminated in the presence of PI (Fig. 6C, lanes 2 and 3). In contrast, PI did not significantly affect the Elf-1–c-Jun interaction (Fig. 6C, lanes 5 and 6). Taken together, these experiments were consistent with a model in which both Ets and NF-κB/NFAT proteins, as well as an appropriate DNA molecule, are required for the formation of a ternary complex containing all three molecules. As has been described previously, the reticulocyte and bacterial lysates used in the binding experiments contain small amounts of DNA molecules, which may allow complex formation in the absence of added DNA (51).

As a further test of the importance of a DNA molecule containing intact Ets and NF-κB binding sites for the formation of the Elf-1–p50 ternary complex, we incubated GST–Elf-1 with ^{35}S-labeled in vitro-translated p50 in the presence of either a wild-type HIV-2 enhancer fragment containing intact NF-κB and Ets binding sites or a mutant oligonucleotide containing a mutation in the NF-κB binding site but an intact Ets binding site (mκB). GST–Elf-1–p50 binding was significantly increased by the addition of increasing amounts of the wild-type HIV-2 DNA (Fig. 7, lanes 2 through 5) but not by the HIV-2 mκB DNA (Fig. 7, lanes 6 through 9). Thus, using three different experimental protocols, we demonstrated that both Ets and NF-κB/NFAT proteins, as well as an appropriate DNA molecule, are required for the formation of a ternary complex containing all three molecules. These findings raised the possibility that the observed physical association between Ets and NF-κB/NFAT proteins simply reflected the simultaneous binding of both proteins to a single DNA molecule. However, this was shown not to be the case in the chimeric protein experiments shown in Fig. 4B. Thus, for example, chimeric NF-κB molecules such as p50/p65 chimeras 1 and 5 were shown by EMSA to bind to DNA with high affinity. However, these molecules failed to associate with Ets proteins. Therefore, DNA binding is necessary but not sufficient for the Ets-NFAT/NF-κB interaction. Several models that might account for this type of interaction are described in detail below (see Discussion).

Physical interactions between Ets and NF-κB proteins are required for synergistic transcriptional activation of the HIV-1 and HIV-2 enhancers. It was shown previously that both Ets and NF-κB proteins can independently activate transcription from reporter constructs containing the HIV-1 or HIV-2 enhancers (38, 40, 62, 71, 86). To determine if Ets and NF-κB proteins can cooperatively activate transcription in vivo and, if they can, whether physical interaction between Ets and NF-κB is required for this activity, COS cells were transfected with a luciferase reporter construct containing a single copy of the intact HIV-1 enhancer in conjunction with various combinations of eukaryotic expression vectors encoding different Ets and NF-κB proteins (Fig. 8A). COS cells rather than T cells were used in these experiments because T cells express high endogenous levels of both NF-κB and Ets proteins. Overexpression of either Ets-1 or NF-κB (p50 + p65) alone resulted in approximately fivefold transactivation of the HIV-1 reporter construct. In contrast, cotransfection of Ets-1 and NF-κB expression constructs resulted in 18-fold transactivation. Thus, Ets-1 and NF-κB can synergistically induce HIV-1 transcription. To determine the role of the physical interaction between Ets and NF-κB in mediating this cooperative effect, we assessed the effect of overexpressing the p50/p65 chimera 5 full-

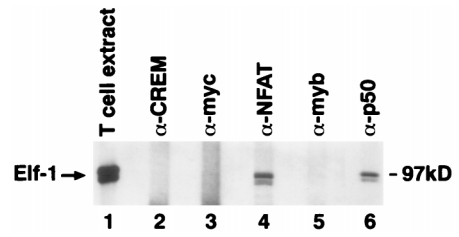

FIG. 5. Elf-1 associates with p50 and NFAT proteins in activated normal human T cells. Cell extracts from activated human peripheral T cells were immunoprecipitated with antibodies specific for CREM, Myc, NFATp/c, Myb, or p50. Immunoprecipitates were denatured, fractionated by SDS-PAGE, and analyzed by protein immunoblot analysis with an α-Elf-1 MAb. Whole T-cell extract (T cell extract) was electrophoresed in parallel on the same gel. The 97-kDa size marker is shown to the right of the autoradiogram.

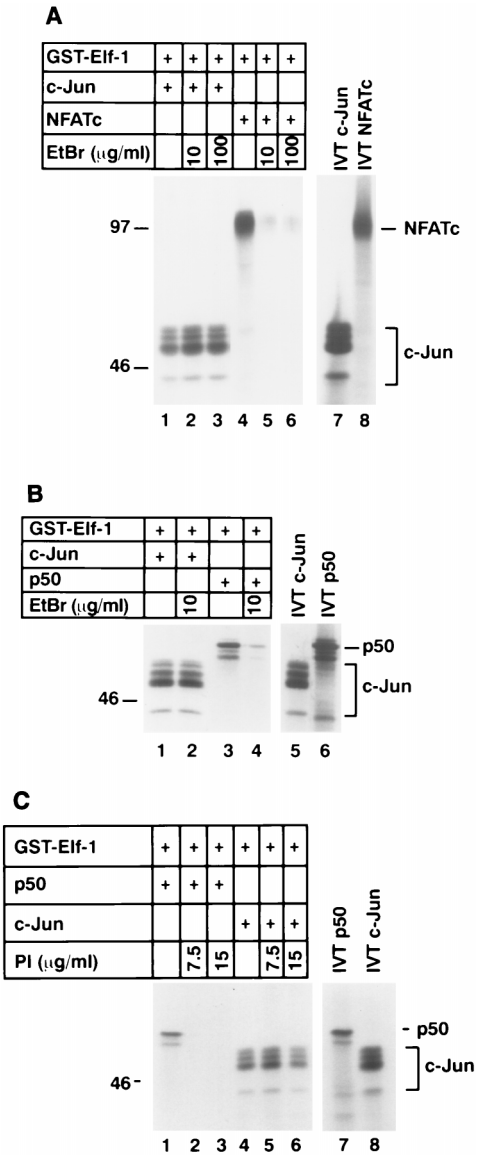

FIG. 6. (A) EtBr specifically abrogates the Elf-1–NFATc association. Equivalent molar amounts of ^{35}S-labeled in vitro-translated (IVT) c-Jun and NFATc were mixed with 1 μg of bacterially expressed GST–Elf-1 in the absence or presence of either 10 or 100 μg of EtBr per ml. (B) EtBr specifically abrogates the Elf-1–p50 association. Equivalent molar amounts of ^{35}S-labeled in vitro-translated c-Jun and p50 were mixed with 1 μg of bacterially expressed GST–Elf-1 in the absence or presence of 10 μg of EtBr per ml. (C) PI specifically abrogates the Elf-1–p50 association. Equivalent amounts of ^{35}S-labeled in vitro-translated p50 and c-Jun were mixed with 1 μg of bacterially expressed GST–Elf-1 in the absence or presence of either 7.5 or 15 μg of PI per ml. The protein complexes were denatured and separated by SDS-PAGE prior to autoradiography. ^{35}S-labeled in vitro-translated proteins were run directly in parallel on the same gels. Size markers (in kilodaltons) are shown to the left of each autoradiogram.

length protein in conjunction with Ets-1 and wild-type NF-κB on HIV-1 enhancer-mediated transcription. The p50/p65 chimera 5 protein retains the abilities to bind to DNA and to associate with p65 but fails to associate with Ets proteins because the 31-aa C-terminal Ets binding domain of p50 has been replaced with the homologous region of p65 (Fig. 4, lane 5). Cotransfection of the p50/p65 chimera 5 protein significantly decreased Ets-1/NF-κB (p50 + p65)-mediated transcription from the HIV-1 enhancer. The resulting level of transcription was approximately equivalent to the level observed following overexpression of Ets-1 alone. It was suggested previously that p50 homodimers can repress transcription from NF-κB sites. To rule out such a direct inhibitory effect by the p50/p65 chimeric protein, we cotransfected equal amounts of wild-type p50 in combination with Ets-1 and NF-κB. In contrast to the inhibition observed following overexpression of the p50/p65 chimera 5 protein, overexpression of wild-type p50 resulted in a slight increase in transactivation (20-fold), suggesting that the inhibition mediated by the p50/p65 chimera 5 protein was specifically due to the inability of this protein to interact with Ets-1. In similar experiments, overexpression of both Ets-1 and NF-κB (p50 + p65) in COS cells resulted in an approximately threefold induction of the HIV-2 reporter construct. This induction required binding sites for both NF-κB and Ets-1 proteins, since mutations in either binding site abolished the activation of the reporter construct following coexpression of both transcription factors (Fig. 8B). This result is consistent with the in vitro binding experiments that demonstrated that binding sites for both NF-κB and Ets proteins are required for their physical interaction.

DISCUSSION

In this report, we have demonstrated a physical interaction between Ets and NF-κB/NFAT transcription factors that is required for the activation of the HIV-1 and HIV-2 enhancers. This interaction is mediated by the DNA binding domains of the two proteins: the Ets domain of the Ets proteins and the C-terminal region of the Rel HD of some, but not all, NF-κB/NFAT proteins. The binding sites on both proteins have been highly conserved during vertebrate evolution. Thus, each of the distantly related Ets proteins Ets-1, Elf-1, and PU.1 retains the ability to interact with multiple, distantly related NF-κB/NFAT proteins, including p50, c-Rel, NFATc, and NFATp. This degree of evolutionary conservation suggests an important function for this protein-protein interaction in regulating mammalian gene expression. The association between NF-κB/NFAT and Ets proteins also requires a DNA molecule in that it is inhibited by EtBr and PI and facilitated by exogenous DNA containing NF-κB and Ets binding sites. Thus, this association involves (at minimum) a tripartite complex composed of an appropriate DNA molecule with bound Ets and NF-κB/NFAT proteins.

The role of DNA in the Ets–NF-κB/NFAT interaction. Our studies demonstrate that DNA binding is necessary but not sufficient for the observed physical interactions between Ets

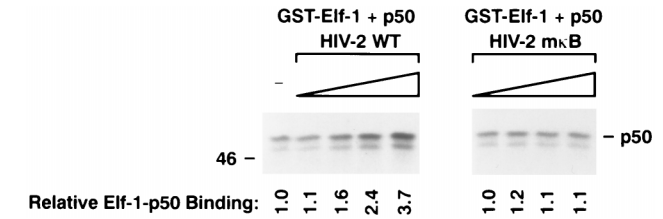

FIG. 7. GST–Elf-1–p50 binding is increased by the addition of DNA containing binding sites for both proteins. ^{35}S-labeled in vitro-translated p50 was incubated with 60 ng of bacterially expressed GST–Elf-1 in the presence of no DNA (lane 1) or of 35 ng (lane 2), 70 ng (lane 3), 140 ng (lane 4), or 350 ng (lane 5) of HIV-2 wild-type DNA or of 35 ng (lane 6), 70 ng (lane 7), 140 ng (lane 8), or 350 ng (lane 9) of HIV-2 mκB DNA. The resulting complexes were separated by SDS-PAGE. Size markers (in kilodaltons) are shown on the left. Relative Elf-1–p50 binding, as determined by phosphorimager analysis, is indicated for each reaction.

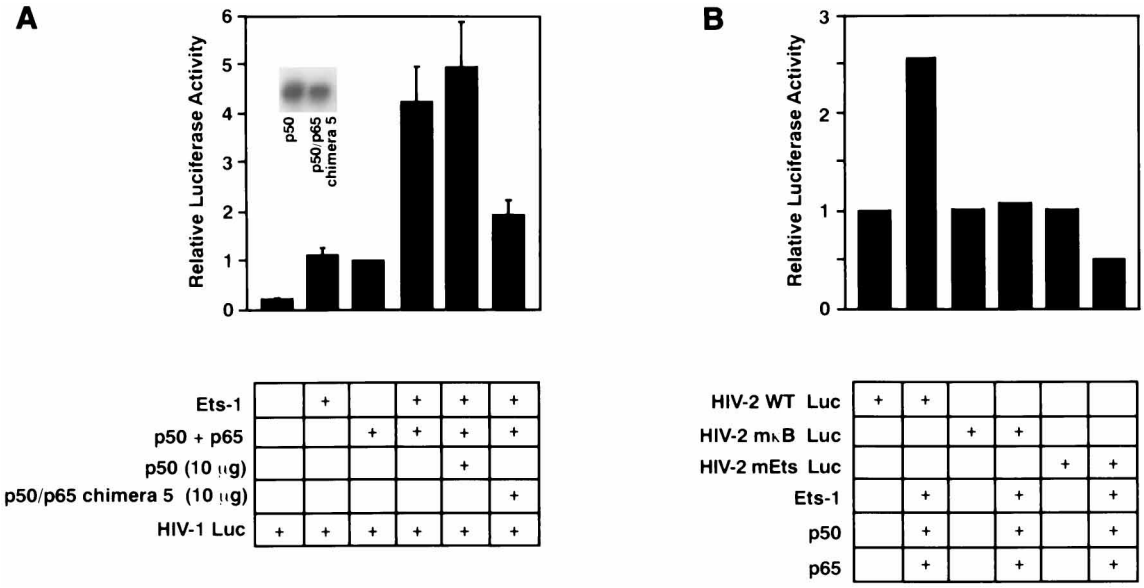

FIG. 8. Cooperative transcriptional activation of the HIV-1 and HIV-2 enhancers by Ets-1 and NF-κB proteins. (A) COS cells were transiently transfected with 1 μg of HIV-1 Luc reporter plasmid and the indicated amounts of Ets-1 and NF-κB expression plasmids. Each transfection mixture contained a total of 21 μg of DNA, including 1 μg of the RSV–β-galactosidase reference plasmid. Cell lysates were prepared 48 h after transfection, normalized for protein content, and analyzed for luciferase and β-galactosidase activities. The data are shown as mean luciferase activity (± standard error of the mean) relative to that observed following transfection of the same cells with the HIV-1 Luc reporter plasmid and p50 and p65 expression plasmids after correction for differences in transfection efficiencies. In vitro-translated p50 and p50/p65 chimera proteins were also assayed for DNA binding activity by EMSA, using a radiolabeled oligonucleotide probe from the HIV-2 enhancer. (B) COS cells were transiently transfected with 1 μg of the HIV-2wtLuc, HIV-2mκBLuc, or HIV-2mEtsLuc reporter plasmids along with 3 μg of Ets-1 and 1 μg each of the NF-κB p50 and NF-κB p65 expression plasmids. Each transfection contained a total of 12 μg of DNA, including 1 μg of the RSV–β-galactosidase reference plasmid. Cell lysates were prepared 48 h after transfection, normalized for protein content, and analyzed for luciferase and β-galactosidase activities. The data are shown as luciferase activity relative to that observed following transfection of the same cells with the appropriate HIV-2 Luc reporter plasmid alone after correction for differences in transfection efficiencies.

and NF-κB/NFAT proteins. In this regard, our results differ significantly from those of a previous study that reported that EtBr does not disrupt a physical interaction between NF-κB p50 and Elf-1 (44). The reason for this difference is unclear. However, it should be emphasized that we have confirmed our findings using multiple concentrations of two different DNA intercalating agents (EtBr and PI) and have also shown a dose-dependent increase in Ets–NF-κB association in the presence of DNA containing adjacent Ets and NF-κB binding sites. Furthermore, the observed Ets–NF-κB interaction does not simply reflect simultaneous binding of both proteins to a single DNA molecule, because chimeric p50/p65 proteins that bind to DNA with high affinity fail to interact with Ets proteins (Fig. 4B). Several possible models might account for the role of DNA in the Ets–NF-κB interaction. First, the DNA (with appropriate cognate binding sites) might serve to increase the local concentrations of the two proteins, thereby facilitating their interactions. In such a model, both protein-DNA and protein-protein interactions might stabilize the tripartite DNA–Ets–NF-κB/NFAT complex. In a second model, binding of one or both proteins to the DNA might produce conformational changes that expose the binding site(s) for the other protein. In this regard, it is of interest that the sites of protein-protein interactions on both the Ets and NF-κB/NFAT proteins are directly adjacent to or overlap the DNA binding domains of both proteins. These two potential mechanisms are not mutually exclusive. The resolution of these models will await the direct determination of the structure of the Ets–NF-κB/NFAT–DNA ternary complex. Finally, it should be emphasized that our data do not allow us to distinguish models in which Ets and NF-κB/NFAT proteins associate directly from models in which accessory or bridging proteins are required for the interaction.

An inducible T-cell enhancon composed of Ets, NF-κB/NFAT, and AP1 proteins. Multiple inducible T-cell enhancers have been shown to contain adjacent or overlapping functionally important binding sites for Ets, NF-κB/NFAT, and AP1 proteins (18, 42, 44, 66, 87, 101). Thus, it is clear that these proteins play a critical role in regulating inducible T-cell gene expression. It was shown previously that multiple Ets proteins can associate directly with each of the Jun proteins (3, 91). This interaction can occur in the absence of DNA and is mediated by the Ets domains of the Ets proteins and the basic domains of the Jun proteins. Similarly, previous reports have demonstrated physical interactions between NF-κB and AP1 and between NFAT and AP1 (9, 43, 72, 92). Interestingly, the NF-κB–AP1 interaction occurs between the basic domains of Jun or Fos and the Rel HD of p65 but not of p50 (92). When taken together with these previous results, our finding of a functionally important physical interaction between Ets and NF-κB/NFAT proteins is consistent with a model in which the assembly of a multiprotein enhancon composed of Ets, NF-κB/NFAT, and AP1 proteins may be a critical step in activating inducible T-cell gene expression. The assembly of such enhancons would involve both protein DNA interactions and protein-protein interactions. In one such model, for example, the binding of Ets and NF-κB proteins to cognate binding sites on the HIV-1 enhancer could facilitate the DNA-independent binding of AP1 to the enhancer. The assembly of this type of tripartite protein structure might be facilitated by the fact that Jun proteins bind to the p65 subunit of NF-κB, while Ets proteins bind to the p50 (but not to the p65) subunit. Support

for such an enhancon model derives from previous studies that have demonstrated synergistic activation of the HIV-1 enhancer by overexpression of AP1 and NF-κB despite the fact that the HIV-1 enhancer lacks an AP1 binding site (92).

The notion of a multiprotein enhancon that regulates the inducible expression of multiple T-cell genes has a number of attractive features. First, such a model might help to explain how T cells integrate multiple activation signals to precisely regulate coordinate changes in gene expression. For example, the activation of Elf-1 requires both the phosphorylation of the protein (3a) and its release from Rb (103), which is mediated by cyclin-dependent kinases (17, 23, 36, 48). Similarly, the activation of NF-κB requires the phosphorylation and subsequent degradation of IκB (4–6, 26, 94), the activation of NFAT requires the calcium-dependent phosphatase calcineurin (24, 59), and the activation of AP1 involves both the transcriptional up-regulation of the Jun and Fos proteins and their phosphorylation (34, 46, 69, 94). Thus, activation of each of these signaling pathways would be required for the assembly of the intact enhancons, a prediction that is consistent with the known requirement for the activation of multiple signaling pathways in the process of T-cell activation. Second, the large number of potential complexes that can be formed between different Ets, NF-κB/NFAT, and AP1 family members provides a dynamic mechanism for the fine regulation of multiple inducible T-cell genes with a minimum number of distinct transcription factors.

The requirement for DNA in the Ets–NF-κB/NFAT interaction has important implications for the differential regulation of inducible T-cell gene expression. The high degree of conservation of protein-protein interaction sites among multiple Ets, AP1, and NF-κB/NFAT family members suggests that it might be possible to form many different multiprotein complexes in the nuclei of activated T cells. Moreover, the DNA-independent nature of the Ets-AP1 and NF-κB–AP1 interactions suggests that such multiprotein complexes could be formed in solution and could bind to DNA elements containing a single Ets, AP1, or NF-κB/NFAT site. Our finding that the Ets–NF-κB/NFAT interaction requires binding sites for both proteins suggests a mechanism for restricting the assembly of the intact enhancon to DNA elements containing both sites. Moreover, the distinct fine specificities of binding of different Ets and NF-κB/NFAT family members (12, 27, 41, 47, 57, 74, 90, 100, 102, 105) suggest that only a limited number of specific complexes would be able to assemble on individual promoter and enhancer elements, thereby conferring a degree of sequence specificity to the assembly process.

The Ets–NF-κB/NFAT association as a target for immunosuppressive and antiviral therapies. The finding that the Ets–NF-κB/NFAT interaction is required for the activation of the HIV enhancers and that this interaction can be inhibited by a dominant-negative form of NF-κB p50 that binds to DNA but fails to interact with Ets proteins suggests that this protein-protein interaction might represent a novel target for immunosuppressive therapies. More generally, the finding that physical interactions between Ets, AP1, and NF-κB/NFAT proteins are important in regulating inducible T-cell gene expression suggests that molecules that disrupt these interactions may be useful in down-regulating T-cell function in vivo. Such molecules might also be useful anti-HIV reagents.

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