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Raf-1 Kinase Targets GA-Binding Protein in Transcriptional Regulation of the Human Immunodeficiency Virus Type 1 Promoter

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The serine/threonine protein kinase Raf-1 is a component of a conserved intracellular signaling cascade that controls responses to various extracellular stimuli. Transcription from several promoters, including the oncogene-responsive element in the polyomavirus enhancer, the c-*fos* promoter, as well as other AP-1- and Ets-dependent promoters, can be induced by Raf-1 kinase. Previously, we have shown that activated Raf-1 kinase transactivates the human immunodeficiency virus type 1 (HIV-1) long terminal repeat and have identified the NF-κB binding motif as a Raf-1-responsive element (RafRE). We now report that Raf-1 kinase-induced transactivation from the HIV RafRE involves the purine-rich-repeat-binding protein (GABP), which is composed of two distinct subunits (α and β). GABP α is an Ets oncogene-related DNA-binding protein, and GABP β contains four ankyrin-like repeats that have been shown to be essential in protein-protein interactions. In electrophoretic mobility shift assays using nuclear extracts from human Jurkat T cells, a protein-DNA complex which was supershifted with antiserum against GABP α and GABP β was observed. Purified recombinant GABP α and β interact with the HIV RafRE as judged from DNA binding assays. Cotransfection experiments with GABP α and β and Raf-1 kinase demonstrate synergistic transactivation of the HIV-1 promoter. Point mutations in the HIV RafRE abolished the Raf-1 kinase- as well as GABP α- and β-induced transactivation. The observed Raf-1–GABP synergism presumably involves phosphorylation of GABP subunits, as treatment of cells with Raf-1 kinase activators serum and 12-*O*-tetradecanoylphorbol-13-acetate increases phosphorylation of GABP in vivo. However, GABP is not a target of Raf-1 kinase; instead, it is a substrate of mitogen-activated protein kinase (MAPK/ERK), since in vitro phosphorylation of GABP α and β was achieved by the reconstituted protein kinase cascade but not with purified Raf-1 or MEK. These results suggest that Raf-1 kinase-induced activation of the HIV-1 promoter is mediated by the classical cytoplasmic cascade resulting in MAPK/ERK-mediated phosphorylation of GABP α and β. Because the HIV RafRE corresponds to a region within the promoter which is essential for regulation of HIV-1 expression, the data indicate that in addition to NF-κB, GABP transcription factors are important for induced expression of HIV.

The long terminal repeat (LTR) of human immunodeficiency virus type 1 (HIV-1) contains multiple regulatory elements mediating viral gene expression. Transcriptional control involves a complex interplay between cellular and viral regulatory proteins and their target sequences within the HIV LTR. Infection of cells by HIV is often characterized by a long period of latency which is thought to be determined at least in part by the availability of host transcription factors (21, 24, 35). External stimuli such as cytokines that activate T lymphocytes or monocytes enhance the replication of HIV in vitro (23). These data indicate that one or more cytokine-regulated transcription factors are important for the expression of viral genes. Proteins belonging to the NF-κB/Rel family of transcription factors and perhaps other proteins which bind the NF-κB motif appear to be important stimulating factors, since the two NF-κB binding sites in the modulatory region of the HIV LTR are critical for the activity of the HIV-1 promoter (16, 35, 40).

Work in several laboratories has delineated a signal transduction pathway from growth factor receptors to the nucleus in which the cytosolic serine/threonine-specific protein kinase, Raf-1, plays an essential role (38). Stimulation of growth factor or cytokine receptors results in the recruitment of Ras guanine nucleotide exchange factor to the activated receptor, activation of Ras, and the subsequent activation of Raf-1 (13, 36, 51). Activated Raf-1 kinase phosphorylates and activates MAP kinase kinase (MEK), which in turn phosphorylates and activates mitogen-activated protein kinase (MAPK)/extracellular regulated protein kinase (ERK). Activated MAPK/ERK is then translocated into the nucleus, where it phosphorylates and thereby regulates several transcription factors (39). Among the direct targets of MAPK/ERK that have been identified are the Ets family transcription factors Elk-1 and Sap-1 (26, 28, 34). Phosphorylation of Elk-1 by ERK is probably involved in serum-induced transcription from the serum-responsive element in the c-*fos* promoter, as point mutations in the MAPK/ERK phosphorylation sites of Elk-1 impaired the serum response (26, 45, 46).

Expression of v-Raf or activated versions of Raf-1 kinase transactivate expression from the oncogene-responsive element in the polyomavirus enhancer (48), from the c-*fos* promoter (27), from serum-responsive elements (37), and from genes driven by AP-1 and Ets binding motifs (8). Raf-1 kinase

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may also be involved in the signaling pathways that lead to activation of the NF-κB/Rel family of transcription factors, although the connection is presumably indirect. We and others showed that the Raf-responsive element (RafRE) corresponds to the NF-κB binding motif within the HIV-1 promoter (9, 20). A set of point mutations in both NF-κB sites blocked the ability of Raf-1 kinase to transactivate HIV LTR-driven expression, indicating that binding to these sites is affected by Raf-1-induced signaling (9). As NF-κB did not appear to be a direct target of the classical Raf/MEK/ERK pathway (9a), other factors had to be considered. Inspection of the NF-κB motif in the HIV LTR revealed that the 3′ half-site of the NF-κB motif contains a purine-rich core sequence motif 5′-GGAA-3′, which represents a potential binding site for transcription factors belonging to the Ets family. Since we have previously shown that activated Raf-1 kinase stimulates expression of a reporter construct driven by two Ets motifs (8), we decided to investigate whether the GA sequence-binding protein (GABP) might be involved in Raf-1 kinase-induced HIV-1 expression.

GABP differs from other Ets family transcription factors in that one subunit contains specific domains for protein-protein interactions reminiscent of the NF-κB/IκB interaction (32). GABP is a heteromeric transcription factor that is composed of two subunits, α and β (Fig. 1C) (32, 33). This transcription factor complex was originally purified from rat liver cells, and biochemical studies of GABP have highlighted a number of molecular characteristics critical for transcriptional activation of herpes simplex virus type 1 (HSV-1) immediate-early genes (32, 33, 44). The 52-kDa GABP α subunit shows significant homology to the DNA binding domains of transcription factors of the Ets family and facilitates weak binding to DNA, as shown on the promoter of the HSV-1 ICP4 gene (32, 47). The 42-kDa β subunit of GABP contains four repeats of a sequence present in several transmembrane proteins, including Notch of fruit flies; ankyrin; and several proteins regulating the activity of NF-κB (15). These amino-terminal repeats of GABP β mediate stable interaction with GABP α and, in a heteromeric complex with GABP α, directly contact their specific DNA substrate (43). In addition, GABP binds to and activates transcription from element 1 in the adenovirus E1A enhancer (2). The human homolog of GABP is the HeLa cell E4TF1 protein, which activates the E1A promoter (49). GABP also controls basal transcription of the TATA-less cytochrome *c* oxidase subunit IV (COXIV) gene promoter and controls the inducible activity of a distal interleukin-2 enhancer (1, 11). Unlike most other characterized Ets family transcription factors, GABP is constitutively expressed in most cell types (32). In this study, we provide the first evidence that Raf-dependent signal transduction communicates with the nuclear target GABP in transcriptional activation of the HIV-1 LTR.

MATERIALS AND METHODS

Expression and reporter plasmids. The current studies employ three Raf-1 expression constructs. Raf-BXB carries a large in-frame deletion of amino acids 26 to 302. This deletion renders Raf-1 constitutively active in NIH 3T3 cells with regard to transformation and induction of AP-1/Ets-driven promoter activity (8, 25, 30). The construct Raf-BXB-301 is identical to Raf-BXB except for the substitution of a W for a K residue at position 375 in the ATP binding site of c-Raf-BXB, which reduces the kinase activity. Raf-C4B is a dominant-negative carboxy-terminal deletion mutant of Raf-1 and contains the Ras binding domain (8). Expression vector sRSV-GABPα was generated by ligating the *Spe*I-*Kpn*I repaired fragment from KS-GABPα into the *Spe*I-*Sma*I site of pSRSPA. sRSV-GABPβ1 was made by ligating the *Spe*I-*Xho*I fragment from KS-GABPβ1 into the *Spe*I-*Xho*I site of pSRSPA. pSRSPA is an expression vector with the Rous sarcoma virus promoter and simian virus 40 polyadenylation signal in a Bluescript backbone (18). Bacterial chloramphenicol acetyltransferase (CAT)-containing plasmids with deletions and mutations in the HIV promoter and the pRSPA-HIV-tat plasmid have been described previously (9).

Cells and metabolic labeling. Human embryonic kidney 293 cells and monolayer cultures of NIH 3T3 cells were routinely maintained in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum (heat inactivated at 56°C for 30 min) at 37°C in humidified air with 5% CO_2. Metabolic labeling of 293 cells with $^{32}P_i$ was conducted by incubating cells (10^6 cells per ml) in phosphate-free medium at 37°C for 2 h. The concentration of isotope added to the medium was 500 μCi/ml. At the end of the 2-h labeling period, cultures were washed twice with phosphate-buffered saline.

DNA transfection. Transfections of NIH 3T3 cells were performed by the calcium phosphate coprecipitation method described by Wigler et al. (50), and 293 cells were transfected by a modified procedure according to the Stratagene transfection protocol. Cells (10^6 per 10-cm-diameter dish) were seeded 1 day prior to transfection. NIH 3T3 cells were transfected with 1 μg of reporter construct, 5 μg of expression vector, 0.5 μg of pRSPA-HIV-tat expression vector, and salmon sperm DNA such that the final concentration of DNA was 20 μg per transfection. After an overnight incubation, the cells were washed and then incubated in media containing 0.3% fetal calf serum. Total cell extracts were prepared 48 h later, and CAT enzyme levels were assayed by a diffusion-based CAT assay as described previously (8). Transfections for CAT assays were performed at least four times with different plasmid preparations.

EMSA. Double-stranded oligonucleotide probes for electrophoretic gel mobility shift assays (EMSAs) were labeled in a reaction mixture containing 200 ng of double-stranded DNA probe, [α-^{32}P]dCTP, 1 mM dATP, 1 mM dGTP, 1 mM dTTP, 500 mM Tris-HCl (pH 7.5), 100 mM $MgCl_2$, and 2 U of Klenow fragment. After a 30-min incubation at 37°C, oligonucleotides were separated on a G-25 Sephadex spin column and finally resuspended in Tris-EDTA (TE) (15,000 cpm/μl). For typical binding reactions, 2 to 5 μg of recombinant protein was incubated at room temperature for 20 min in the absence or presence of competitor DNA in a 23-μl reaction mixture containing 25 mM Tris (pH 7.5), 1 mM EDTA, 0.5 mM DDT, 100 mM KCl, 0.1% (vol/vol) Nonidet P-40, 1 μg of bovine serum albumin, 10% (vol/vol) glycerol, and 0.5 μg of poly(dI-dC); 30,000 cpm of labeled oligonucleotide was added, and the mixture was subsequently loaded on a 5% nondenaturing polyacrylamide gel equilibrated with 0.25× Tris-borate-EDTA (TBE) and electrophoresed for 4 to 6 h at 150 V at 4°C. Gels were then dried and visualized by autoradiography. In the experiments shown in Fig. 1A, the reaction mixture was incubated with 1 μl (1.0 μg/μl) of antiserum for 10 min at room temperature and control experiments were performed with preserum and anti-Max (C-17; Santa Cruz) antibody.

Recombinant proteins and antiserum. GABP α and GABP β recombinant proteins were prepared according to the method of Thompson et al. (43), with slight modifications. Briefly, GABP α and GABP β were expressed separately by using the phage T7 expression system in *Escherichia coli* BL21(DE3) (42). A bacterial culture was grown to an optical density at 595 nm of 0.6 and induced with isopropyl-β-D-thiogalactopyranoside at 25°C for 4 h. The cell lysate supernatant was applied to an 80-ml Q-Sepharose Fast Flow (Pharmacia) anion-exchange column and eluted with a 75 to 500 mM NaCl gradient. Peak fractions were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and were used in this study with no further purification. Both proteins were purified to approximately 90% homogeneity by this method. Purified proteins were used to generate rabbit antisera against GABP α and GABP β. Prebleed and sera after each boost were tested in immunoprecipitation and Western blot (immunoblot) analysis using 293 cells transiently transfected with GABP α and GABP β or bacterially expressed GABP proteins. The baculovirus encoding full-length MEK-1 and the bacterial expression vector encoding histidine-tagged ERK-2 were described previously (41). The expression and purification of MEK-1 and histidine-tagged ERK-2 were performed as described by Sithanandam et al. (41).

Kinase assay. Nonactivated bacterially expressed ERK-2 (2.8 μg) was incubated with enzymatically activated, baculovirus-expressed MEK-1 (0.25 μg) in 15 μl of buffer A {20 mM magnesium acetate, 0.8 mM unlabeled ATP, 50 mM Tris-HCl [pH 7.4], 0.1 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid [EGTA], 0.1% [vol/vol] β-mercaptoethanol, 0.1 mM sodium orthovanadate} for 15 min at 30°C. The phosphorylation of GABP subunits was initiated by adding 2.5 μl of recombinant GABP α and GABP β (1.3 μg each per μl) and 12.5 μl of buffer B (50 mM sodium β-glycerophosphate [pH 7.0], 0.1 mM EDTA, 10 mM magnesium acetate, 0.1 mM unlabeled ATP, and 0.1 mM [γ-^{32}P]ATP, 10 μCi/mmol). After incubation of the samples for 20 min at 30°C, the reactions were terminated by boiling the mixtures in Laemmli buffer (Sigma). Each sample was subjected to SDS-PAGE according to the procedure of Laemmli and electroblotted to nitrocellulose BA-S 85 membrane (Schleicher & Schuell). After autoradiography, Western blot analysis was performed for GABP α and GABP β.

Immunoprecipitation and Western blot. Cells were lysed with a modified radioimmune precipitation buffer (25 mM Tris-HCl [pH 8.0] containing 137 mM NaCl, 10% [vol/vol] glycerol, 0.1% [vol/vol] SDS, 0.5% [vol/vol] deoxycholate, 1% [vol/vol] Nonidet P-40, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM Na_3VO_3, 0.15 U of aprotinin per ml, 20 μM leupeptin) at 4°C for 30 min. Cell debris was removed by centrifugation at 20,000 × *g* for 10 min. Supernatant was then incubated with rabbit anti-GABP α or rabbit anti-GABP β immunoglobulin G at 4°C for 2 h. The immunocomplexes were precipitated with protein

A agarose and extensively washed with buffer TLB (20 mM Tris [pH 7.4], 137 mM NaCl, 10% [vol/vol] glycerol, 1% [vol/vol] Triton X-100, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM Na_3VO_3, 0.15 U of aprotinin per ml, 20 μM leupeptin). The immunocomplexes were suspended in electrophoresis sample buffer and heated at 100°C for 3 min. After SDS-PAGE, 10% polyacrylamide gels were electroblotted onto Immobilon polyvinylidene difluoride (PVDF) membrane (Millipore) and subjected to autoradiography, and then immunodetection with GABP-specific antisera was performed. The membranes were incubated in blocking buffer (nonfat dry milk) and washed in TBST (41). As a secondary antibody, horseradish peroxidase-conjugated protein A (Boehringer) was used, after which a standard electrochemiluminescence reaction (ECL kit; Amersham, Buchler GmbH, Braunschweig, Germany) was carried out.

Phosphoamino acid analysis. Phosphoamino acid analysis was performed as described by Bolye et al. (3). ^{32}P-labeled GABP was excised from Immobilon PVDF membrane, and phosphopeptides were hydrolyzed in 6 N HCl at 110°C for 60 min. After lyophilization, the resulting phosphoamino acid samples were resuspended in 10 μl of pH 1.9 buffer (formic acid-glacial acetic acid-distilled water, 50:156:1,794), which contains 15 parts buffer to 1 part cold phosphoamino acid standards (1 mg of P-serine, P-threonine, and P-tyrosine per ml), and applied to cellulose-coated thin glass plates. High-voltage electrophoresis was performed in pH 1.9 buffer for 20 min at 1.5 kV. For the second dimension, electrophoresis was carried out for 16 min at 1.3 kV with pH 3.5 buffer (pyrine-glacial acetic acid-distilled water, 10:100:1,890). ^{32}P-labeled phosphoamino acids were identified by autoradiography, and phosphoamino acid standards were identified by spraying with ninhydrin.

RESULTS

Jurkat cell nuclear extracts contain NF-κB element binding GABP subunits. To investigate the role of GABP in the Raf-dependent signaling pathway leading to HIV-1 promoter activation, we studied whether specific GABP-DNA interactions are present in T-cell nuclear extracts. Nuclear extracts were prepared from 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-stimulated Jurkat cells and incubated with ^{32}P-labeled NF-κB oligonucleotides (1xHIV-κB), and free DNA as well as protein-DNA complexes were resolved in EMSAs. As shown in Fig. 1A, incubation of labeled oligonucleotides with Jurkat nuclear extracts results in protein-DNA complex formation. To demonstrate that these complexes contained GABP subunits, antibody supershift experiments were performed with an antiserum against GABP α or GABP β. In the absence of Jurkat nuclear extracts, the antibodies did not shift the NF-κB probe, and a supershifted band was detectable with GABP α, GABP β, and anti-RelA (p65) antibodies when Jurkat nuclear extracts were used (Fig. 1A and data not shown). In contrast, an antibody specific for a second Ets family transcription factor, Ets-1, did not induce a supershifted band.

GABP α and β subunits can bind to the NF-κB element of the HIV-1 promoter. To demonstrate that GABP can bind directly to the NF-κB sites in the HIV-1 promoter, we performed EMSAs with purified bacterially expressed GABP α and β. EMSAs were performed with an oligonucleotide probe containing HIV-1 promoter sequence between nucleotide positions −110 and −79. As shown in Fig. 1B, when both GABP α and β were used, two distinct protein-DNA complexes (C1 and C2) were detectable in EMSAs. No evidence of protein complexes that bound the RafRE oligonucleotide were seen with either GABP α or GABP β. The HSV-1 ICP4 oligonucleotide probe, originally used to characterize GABP DNA binding (43), exhibited the same binding profile (data not shown). Competition assays demonstrated a dose-dependent competition with wild-type RafRE but not unrelated (AP-1) oligonucleotides (Fig. 1B), indicating the specificity of GABP binding. GABP α and GABP β binding to NF-κB sites was also sensitive to competition with the HSV-1 ICP4 probe oligonucleotides (data not shown). Interestingly, oligonucleotides containing point mutations in the 3′ half-sites of both NF-κB elements were unable to compete for binding, whereas oligonucleotides containing point mutations in both 5′ NF-κB half-sites retain competing activity for GABP α and β complexes (Fig. 1B). We therefore conclude that GABP α and β complexes interact with the Ets core consensus sequence located in the 3′ half-site of the HIV LTR NF-κB elements.

Activated Raf-1 kinase synergizes with GABP in stimulating the HIV-1 promoter activity. To determine if activated Raf-1 was capable of cooperating with GABP in transcriptional activation, NIH 3T3 cells were cotransfected with HIV LTR reporter constructs, either with constitutive active (Raf-BXB) or inactive (Raf-BXB-301) Raf-1 (Fig. 2A) and/or GABP α and/or β expression vectors (Fig. 1C). The results shown in Fig. 2B indicate that cotransfection of Raf-BXB together with reporter plasmid L3BCAT and pRSPA (empty expression vector) results in a ninefold stimulation in comparison with that achieved with cotransfection with Raf-BXB-301, L3BCAT, and pRSPA. Cotransfection of Raf-BXB with either GABP α or GABP β did not significantly increase transcriptional activity. In contrast, cotransfection of both GABP subunits (GABP α and GABP β) with Raf-BXB resulted in a synergistic activation of the L3B-driven gene expression. These data indicate that the serine/threonine kinase Raf-1 activates GABP α- and GABP β-mediated gene expression. The same set of experiments was performed with a reporter construct containing the full-length HIV-1 LTR (L3BCATt), which also results in synergistic transactivation (Fig. 2C). Using the HIV-1 NF-κB sites linked to a heterologous promoter, we have previously shown that the context of the κB binding sites within the HIV LTR was not restricting Raf-1-induced transactivation (9). In order to evaluate the combinatory effect of Raf-1–GABP, the two κB binding sites from the HIV-1 enhancer were used in the c-*fos* minimal promoter context (4). Cotransfection of Raf-BXB and both GABP subunits results in a fourfold higher level of CAT activity relative to that obtained by cotransfection with Raf-BXB and either GABP α or GABP β (Fig. 2D). These results demonstrate that activated Raf-1 can modulate expression from the HIV-κB binding sites via GABP subunits, irrespective of their promoter context.

Effects of point mutations in the NF-κB element of the HIV-1 LTR. Point mutations in the 3′ and 5′ half-sites of the NF-κB elements of the HIV-1 promoter (in an L3BCAT background) abolish the transactivation of the HIV-1 promoter induced by Raf-1 kinase (9). We used this set of L3BCAT reporter constructs containing point mutations in the NF-κB elements (Fig. 3) to determine their effect on the observed Raf-1–GABP synergism. NIH 3T3 cells were cotransfected with both GABP α and GABP β, either with Raf-BXB or Raf-BXB-301 and with different L3BCAT reporter constructs, L3BCAT-M4, L3BCAT-M5, and L3BCAT-M3, respectively (Fig. 3). Point mutations in the 3′ half-site of the NF-κB motif which are thought to be at the target site of GABP abolish Raf-1-induced transcriptional activation of the HIV-1 LTR (Fig. 3), indicating that the intact GABP binding site is required. Interestingly, point mutations in the 5′ half-site of the NF-κB motif also block HIV LTR-driven expression. We conclude that a factor binding to the 5′ half-site, presumably NF-κB, is also important.

In vivo phosphorylation of GABP. Since it became obvious that Raf-1 kinase affects GABP activity, we tested whether GABP is phosphorylated in vivo by Raf-1 kinase activators (serum and TPA) and constitutive versions of Raf-1 kinase. Human embryonic kidney cells were cotransfected with GABP and/or Raf-1 expression vectors and metabolically labeled with $^{32}P_i$, and GABP α and β were immunoprecipitated from cell lysates. The basal phosphorylation level of GABP α and GABP β (Fig. 4A and C, lanes 1) was increased two- to fourfold after stimulation with serum and TPA (Fig. 4A and C, lanes 2 to 4). Interestingly, the kinetics of phosphorylation differ between

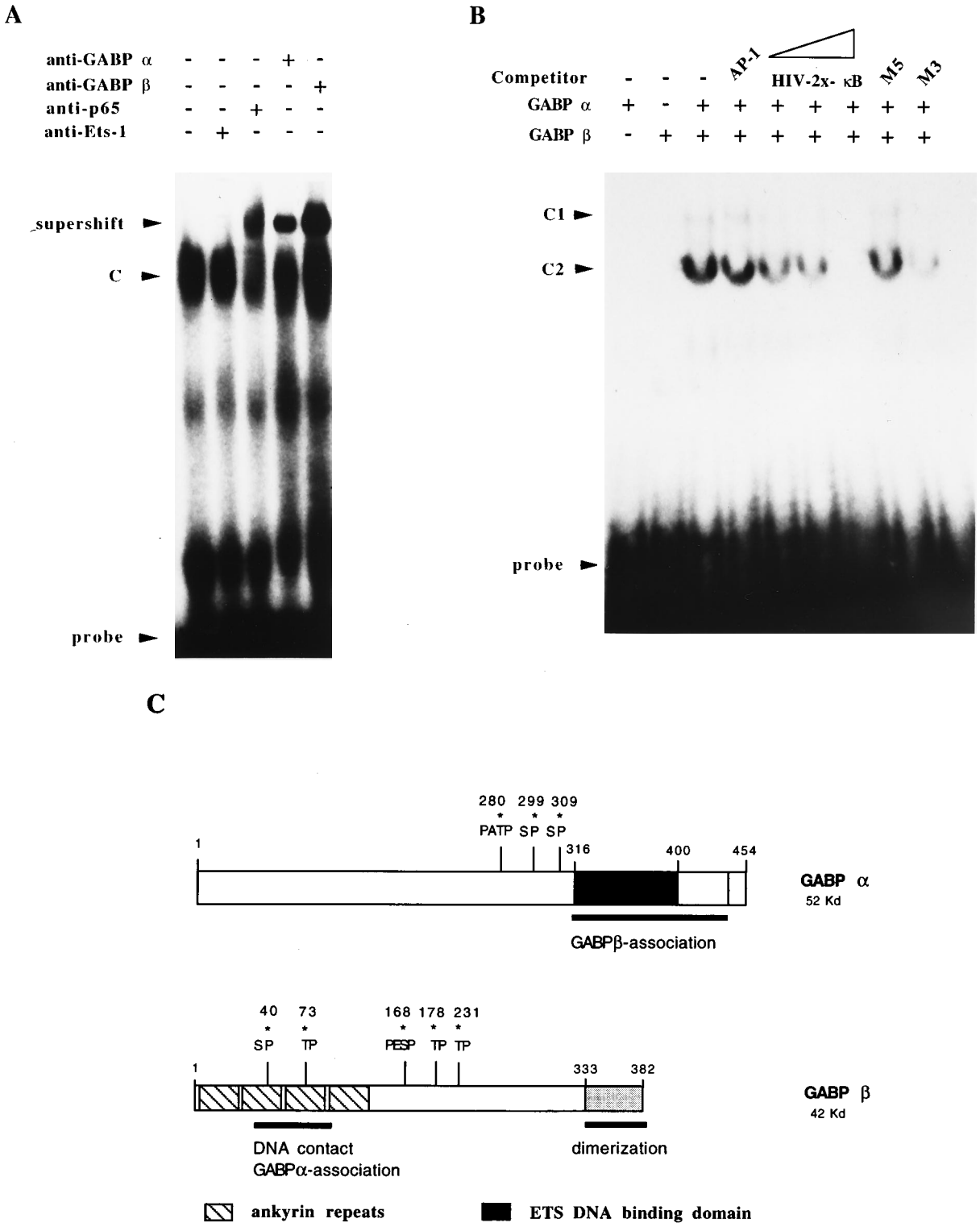

FIG. 1. (A) GABP α and β are present in Jurkat cell nuclear extract. Nuclear extracts from TPA (100 ng/ml, 3 h)-stimulated Jurkat cells were analyzed by EMSAs. A ^{32}P-labeled oligonucleotide (1xHIV-κB site) was used as a probe. After protein-DNA binding, 1 μl of the respective antiserum was added to the reaction mixture and mixtures were analyzed on a 4% native polyacrylamide gel. Anti-GABP α, anti-GABP β, and anti-RelA (p65) antisera supershifted a protein-DNA complex. The supershifted bands are indicated by an arrow. (B) Recombinant GABP α and β subunits bind to the NF-κB binding sites within the HIV-1 promoter. Bacterially expressed and purified GABP α and/or β subunits were incubated with the ^{32}P-labeled HIV-2x-κB oligonucleotide probe (30,000 cpm) and analyzed on a 4% native polyacrylamide gel. Competition assays were performed with the indicated unlabeled wild-type oligonucleotide (HIV-2x-κB) at 50-, 100-, and 500-fold molar excesses and with mutant oligonucleotides (500-fold molar excess) containing point mutations in the 5′ half-site (M3) or 3′ half-site (M5) of the NF-κB element. The AP-1 oligonucleotide was included as a nonspecific competitor at a 500-fold molar excess. The mobilities of GABP protein–HIV-2x-κB oligonucleotide complexes are indicated as C1 and C2, and free oligonucleotides are designated "probe." (C) Schematic representation of GABP. GABP is an Ets family transcription factor that is composed of two subunits (α and β). The α subunit contains the Ets DNA binding domain. The β subunit contains four ankyrin repeat motifs. These repeats have been shown to be essential for GABPα and β dimerization. Regions of GABP that are involved in DNA binding and α-β association are indicated. The positions of consensus ERK phosphorylation sites on GABP subunits (P-x-S/T-P or S/T-P motifs) are shown.

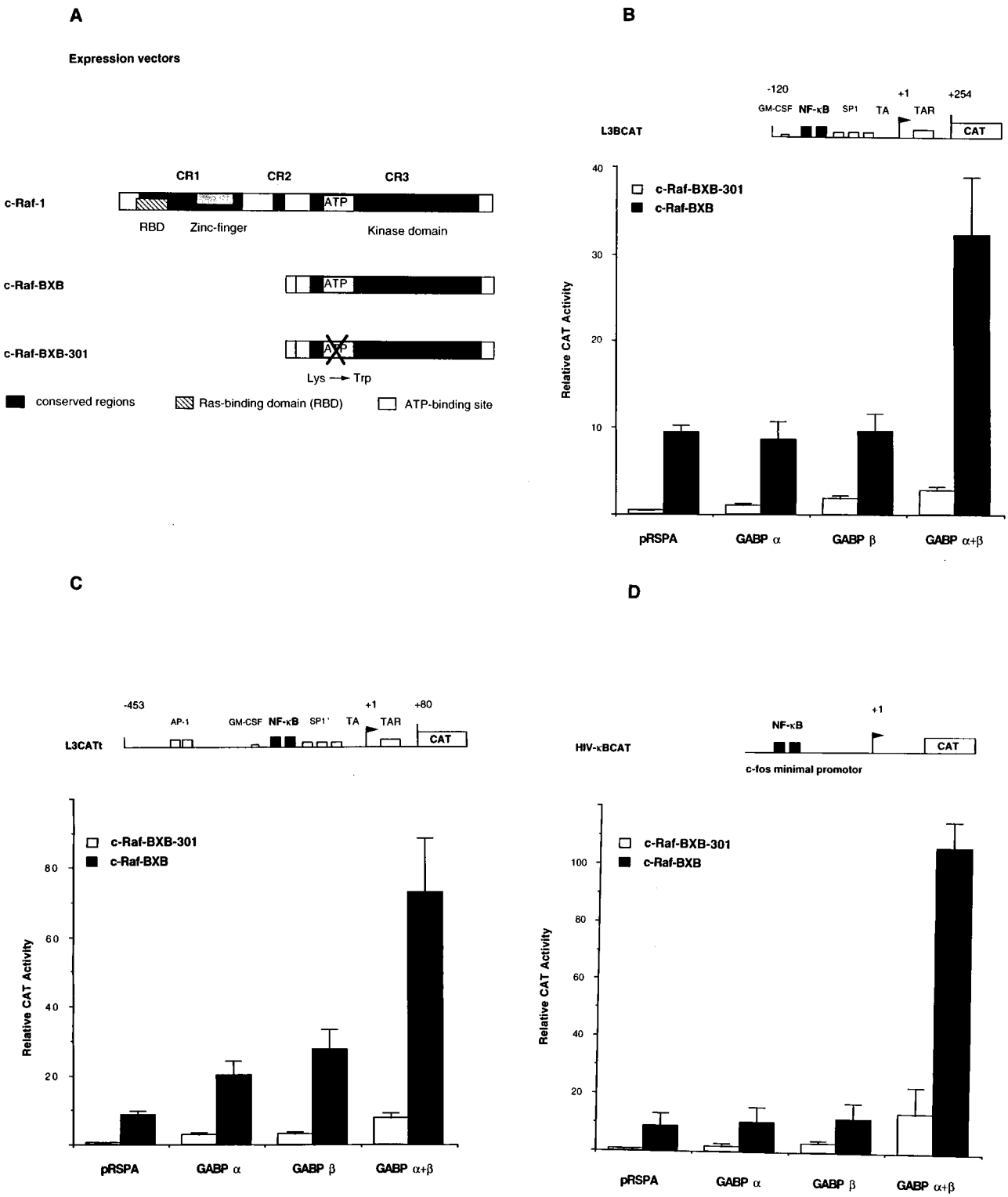

FIG. 2. Raf-1 synergizes with GABP for HIV LTR-driven expression. (A) Schematic representation of Raf-1 expression constructs. The positions of the Raf family conserved regions, CR1, CR2, and CR3, are indicated. c-Raf-BXB is a construct with an in-frame deletion of amino acids 26 to 303 in the regulatory region of c-Raf. This deletion renders Raf-1 constitutively active in regard to transformation and induction of AP-1/Ets-driven promoter activity of NIH 3T3 cells. c-Raf-BXB-301 contains a single amino acid substitution, K to W at position 375, in the ATP binding site of c-Raf-BXB, which disturbs its activity. (B to D) NIH 3T3 cells were transfected with pRSPA (5 μg of empty expression vector), pRSPA-HIV-tat (0.5 μg; panels B and C only), and pRSPA expression vectors (5 μg each) encoding GABP α, GABP β, or both GABP subunits with either inactive Raf-1 (c-Raf-BXB-301) or constitutive kinase-active Raf-1 (c-Raf-BXB). The following CAT reporter constructs were used: L3BCAT (HIV LTR deletion construct containing the RafRE; nucleotides −120 to +254 of the HIV LTR) (B), L3BCATt (HIV LTR full-length promoter construct from nucleotides −453 to +80 of the HIV LTR) (C), and HIV-κBCAT (a c-*fos* minimal promoter reporter construct containing the two κB sites from the HIV-1 enhancer) (D). Cell extracts were harvested 48 h after transfection, and CAT activity was determined as described in Materials and Methods. The standard deviations of the mean values from three independent experiments are indicated by error bars.

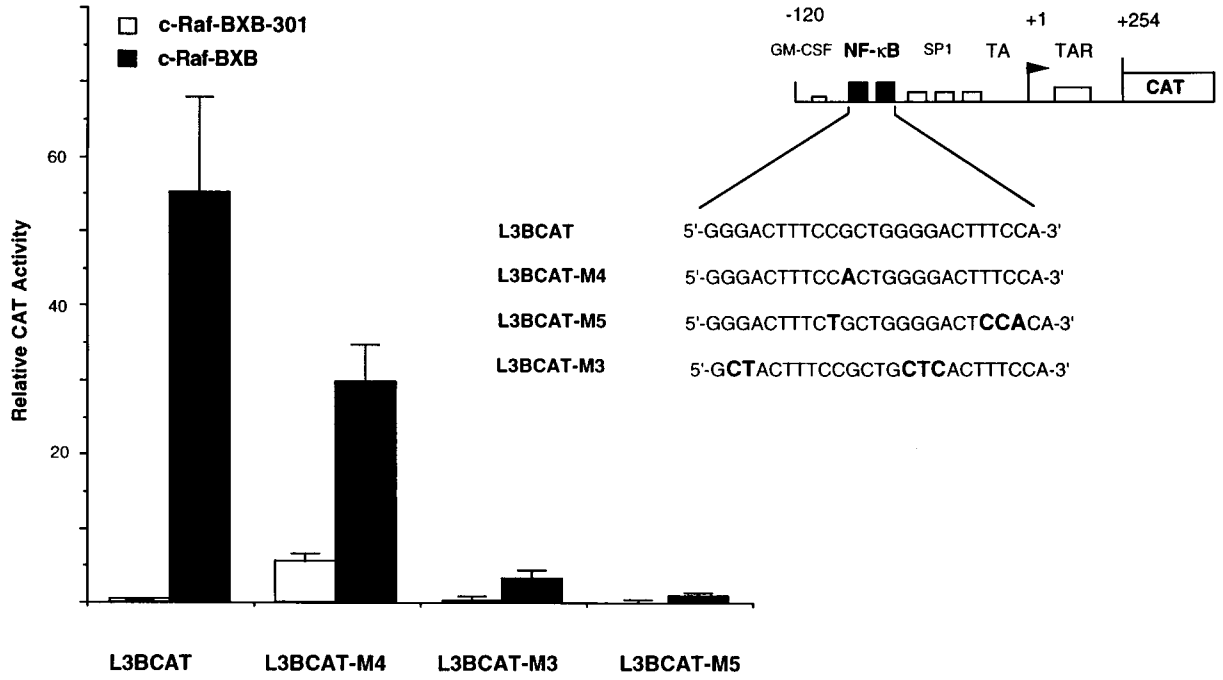

FIG. 3. Point mutations in HIV RafRE block GABP-mediated transcriptional activation of the promoter. Effects of NF-κB element point mutations in the HIV-1 promoter were determined by using the protocol described in the legend to Fig. 2. NIH 3T3 cells were cotransfected with wild-type and mutant L3BCAT reporter constructs, GABP α and GABP β, and either Raf-BXB or Raf-BXB301. Cell extracts were harvested 48 h after transfection, and CAT activity was determined. The standard deviations of the mean values from three independent experiments are indicated by error bars. A schematic illustration of point mutations introduced into the NF-κB element within the HIV-1 promoter (L3BCAT) is shown on the right. Point mutations were introduced by using a PCR-based in vitro mutagenesis strategy (9). The point mutations illustrated were introduced as single (L3BCAT-M4) and double (L3BCAT-M3 and L3BCAT-M5) mutations.

GABP α and GABP β. An increase in GABP α phosphorylation was observed 10 min after stimulation with serum and TPA, whereas the kinetics of GABP β phosphorylation were slower and required 60 min of treatment with serum and TPA to achieve enhanced phosphorylation. Cotransfection of Raf-BXB (Fig. 4A and C, lanes 6) versus cotransfection with a dominant negative version of Raf-1 (Raf-C4B) (Fig. 4A and C, lanes 5) also showed a significant increase in GABP α and GABP β phosphorylation. An unexpected finding in these experiments was the appearance of an additional 40-kDa phosphoprotein which is reactive with GABP α-specific antiserum. This additional phosphoprotein is not detected in Raf-C4B-cotransfected 293 cells.

GABP subunits are phosphorylated by ERK. In order to identify potential kinases of GABP α and β, the bacterially expressed GABP α and β proteins were tested as substrates in in vitro kinase assays (Fig. 5). As activated Raf-1 did not phosphorylate GABP subunits in vitro (data not shown), we focused on Raf-1 kinase downstream activators MEK and ERK. Baculovirus-expressed and enzymatically active MEK-1 was incubated with or without bacterially expressed GABP subunits and/or ERK-2 in buffer containing [γ-^{32}P]ATP and Mg^{2+}. In the reaction mixture containing MEK-1, ERK-2, GABP α, and GABP β, the phosphorylation of the 52-kDa recombinant GABP α and the 42-kDa recombinant GABP β was detected (Fig. 5A, lane 4). Western blotting confirmed the identity of the proteins (Fig. 5B). Similar results were obtained in kinase assays when bacterially expressed ERK-1 was used instead of ERK-2. A kinase-inactive ERK-1 mutant showed no GABP α and β phosphorylation (data not shown).

Phosphoamino acid analysis of GABP subunits. To identify the phosphorylated amino acids present in GABP subunits, ERK-2-phosphorylated GABP α and β proteins were used for phosphoamino acid analysis. Phosphoamino acids were separated by electrophoresis and detected by autoradiography. As shown in Fig. 6A, GABP α protein was phosphorylated only at the threonine residues, whereas GABP β (Fig. 6B) was phosphorylated at serine and threonine residues.

DISCUSSION

We show here that the RafRE in the HIV LTR corresponding to the two NF-κB binding motifs (9) binds the Ets family transcription factor GABP α and β heteromeric complex. GABP presumably mediates, at least in part, the Raf-1 effect on HIV LTR transcription, as it is normally expressed in T cells and coexpression with Raf-1 leads to a synergistic transactivation of the HIV-1 promoter. The functional connection between Raf-1 and GABP involves posttranslational modulations of GABP subunits by the classical cytoplasmic Raf/MEK/ERK cascade as Raf-1 kinase activators serum and TPA stimulate GABP phosphorylation in vivo. Moreover, activated Raf-1, Raf-BXB, increased GABP phosphorylation, and the dominant negative Raf-1 mutant, Raf-C4B, dramatically decreased GABP phosphorylation. In addition, in vitro reconstitution of the kinase cascade results in phosphorylation of GABP α and β on threonine and on serine plus threonine, respectively.

The GABP α and β complex binds to both NF-κB sites within the HIV-1 promoter, as demonstrated by EMSAs. In competition assays an oligonucleotide with mutations in the -GGAA site (M5) was not able to compete, in contrast to oligonucleotides with mutations in the 5′ half-site of the NF-κB motif. The ability of GABP to bind in a cooperative fashion the 5′-GGAA-3′ site as a heteromeric protein has also been observed with other viral and cellular promoter elements. One well-studied system is the promoter region of ICP4, which

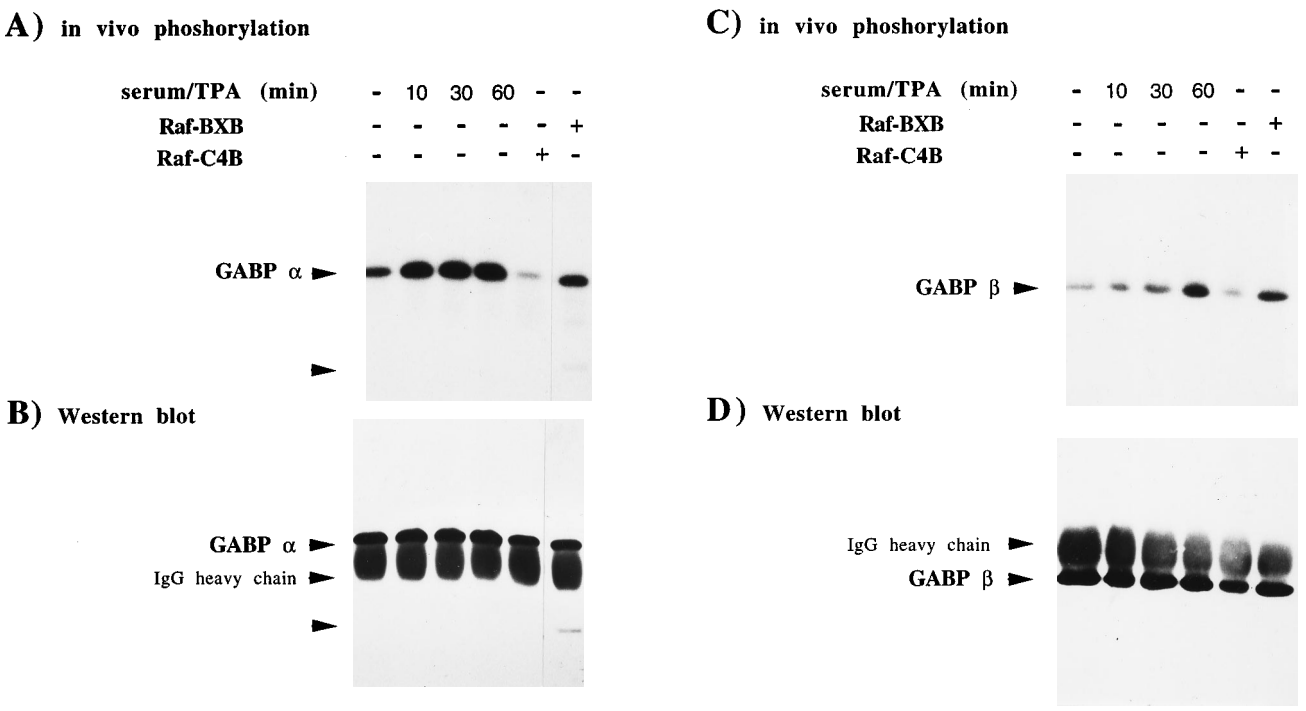

FIG. 4. In vivo phosphorylation of GABP subunits in human embryonic kidney cells. 293 cells were cotransfected with 5.0 or 7.5 μg of expression vectors encoding GABP β or GABP α, respectively. Cells were labeled with $^{32}P_i$ and lysed, and GABP α and GABP β were immunoprecipitated with anti-GABP α and GABP β antisera, respectively. The immunoprecipitates were analyzed by SDS–10% PAGE, electroblotted onto Immobilon PVDF membrane, and analyzed by using a phosphoimager. (B and D) To demonstrate equal protein concentration, a Western blot analysis for GABP subunits was performed as described in Materials and Methods. (A and C) Lanes 2 to 4 were pulsed with 10% serum and TPA (100 ng/ml) for 10 (lanes 2), 30 (lanes 3), or 60 (lanes 4) min after 2 h of ^{32}P labeling; contents of lanes 1 were serum starved and labeled without serum-TPA stimulation; and contents of lanes 5 and 6 were additionally cotransfected with 5 μg of dominant negative Raf-C4B and constitutive active Raf-BXB, respectively. GABP α and GABP β are indicated by arrowheads. IgG, immunoglobulin G.

is an immediate-early gene of HSV-1 (44). The control of the ICP0 and ICP4 HSV immediate-early genes is critical for the determination of the course of viral lytic or latent infection (19). Two distinct *cis*-regulatory motifs are present in this promoter. One motif is the nonanucleotide sequence 5′-TAAT-GARAT-3′, which binds the cellular transcription factor Oct-1 and the viral transactivating factor VP16. The other motif is the purine-rich hexanucleotide sequence 5′-GCGGAA-3′. GABP binds to the second site as a heterotetrameric protein and synergizes with the factors bound to the first site, involving complex stabilization (19). Interestingly, the two 5′-CCG-GAA-3′ GABP binding sites in the ICP4 enhancer form a direct tandem repeat, whereas in the HIV-1 promoter the -GGAA- sites within the NF-κB binding element are separated by more than 9 nucleotides. In the adenovirus E1A enhancer, the binding sites for GABP are critical elements that regulate E1A transcription in vivo (6). GABP binds cooperatively to two of the GABP binding sites in the E1A enhancer region that are spaced 30 bp apart. However, cooperative binding was not affected by deletions of 6, 7, or 13 nucleotides between the two sites (2, 7). Thus, there is considerable flexibility in the spacing of GABP binding sites in various promoters. The arrangement of these binding sites may have functional consequences for interaction between protein complexes in the composite promoter.

M4 contains point mutations in the 3′ half-site of the upstream NF-κB binding element and reduces but does not abolish Raf-1–GABP cooperation. Point mutations in both NF-κB elements, in the 3′ as well as the 5′ half-sites of the two NF-κB sites, abolish Raf-1–GABP synergy. GABP binding correlates with transcriptional activity of the HIV-1 promoter, as muta-

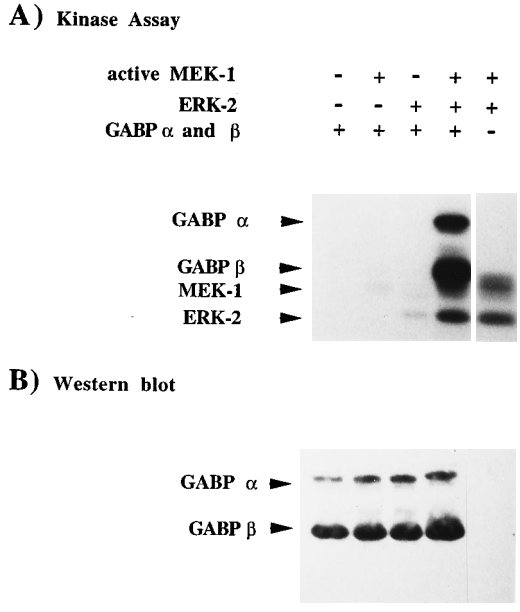

FIG. 5. Activated ERK phosphorylates GABP α and GABP β in vitro. (A) Kinase assay. Baculovirus-expressed and enzymatically active MEK-1 was incubated with and without bacterially expressed GABP α and GABP β and/or recombinant ERK-2 in buffer containing [γ-^{32}P]ATP and Mg^{2+}. Details are described in Materials and Methods. (B) Immunoblot. The phosphorylated bands correspond to GABP α and GABP β as determined by Western blot analysis using GABP α- and GABP β-specific antisera.

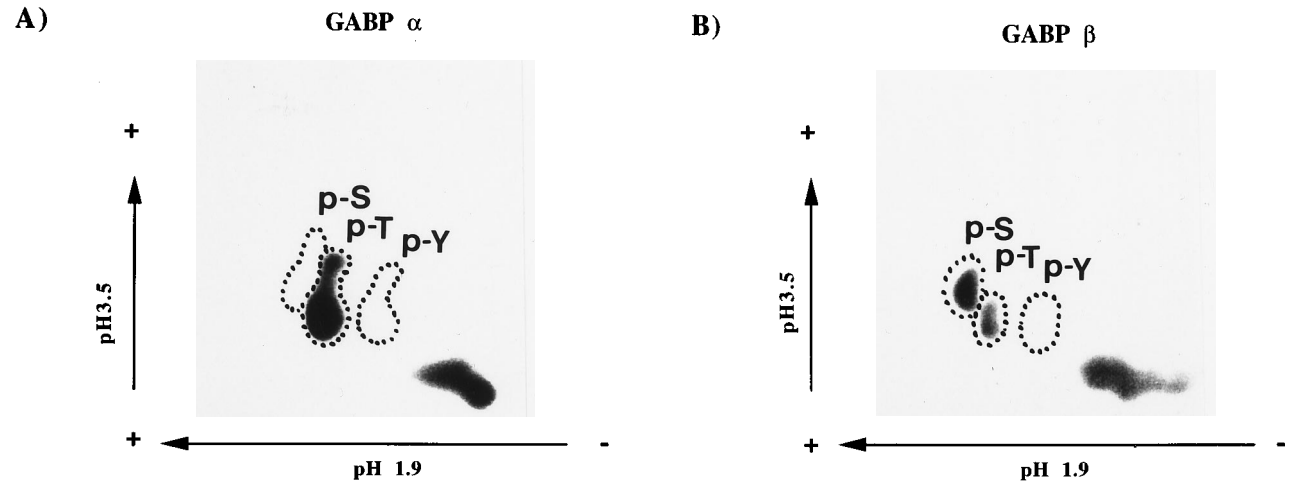

FIG. 6. Phosphoamino acid analysis of GABP subunits. ERK-phosphorylated GABP α (A) and GABP β (B) were separated by SDS–10% PAGE and electroblotted onto Immobilon PVDF membrane, and GABP α and β bands were excised and hydrolyzed. Phosphoamino acids were separated by two-dimensional electrophoresis and autoradiographed.

tions in the 3′ NF-κB half-site abolish GABP binding in vitro and Raf-1-induced transactivation in vivo but do not affect binding of c-Rel or NF-κB1 (data not shown). However, GABP binding does not appear to be affected by ERK-2-induced phosphorylation of GABP subunits in vitro (data not shown). Since mutations in the 5′ NF-κB half-site were also inactivating, it has to be considered that cooperative binding between GABP and proteins of the NF-κB/Rel family may take place. This would indicate a convergence of distinct signaling pathways (the NF-κB pathway and the Raf-1/MEK/ERK pathway) on this promoter element. The opportunity for such a cooperation exists in Jurkat cells, as in studies with anti-p65 antibody the complex from stimulated Jurkat cell nuclear extracts was supershifted (Fig. 1). Protein-protein interactions between Ets factors and neighboring transcription factors may be a common mechanism (12, 22) and may include GABP transcription factor. For example, the Ets family member Elk-1 has been demonstrated to bind cooperatively with the serum response factor to the SRE sequence element of the c-*fos* gene promoter (28). One domain for interaction is the so-called B-box in Ets factors Elk-1 and Sap-1 (12, 29). However, the Ets-related GABP α does not have the B-box domain, and we therefore have to consider other motifs, such as the ankyrin-like repeats on GABP β. It has been noted for Bcl-3, a nuclear transcription factor which also contains ankyrin-like repeats, that these domains mediate interaction with other members of the Rel family of transcription factors (5).

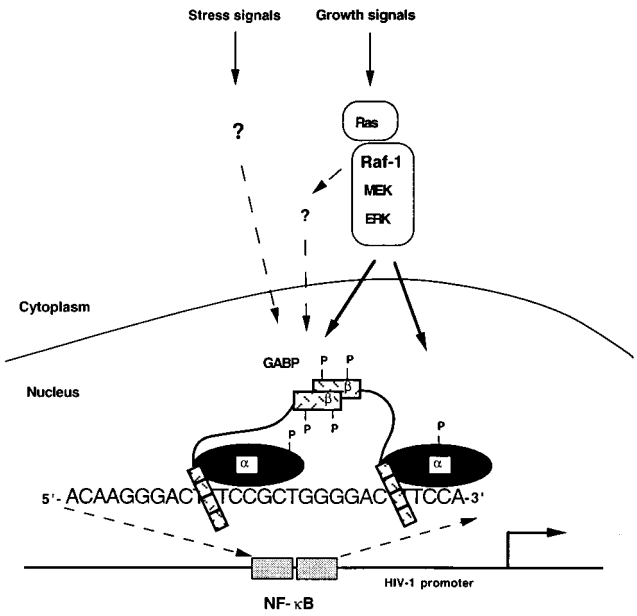

FIG. 7. Schematic model for Raf-1-induced transactivation of the HIV LTR. Following activation, Raf-1 induces the classical cytoplasmic ERK cascade resulting in the phosphorylation of GABP α and β. This complex functions to activate transcription from the NF-κB binding sites within the HIV LTR. The putative contribution by independent signaling pathways is indicated by dotted lines.

Our study has identified the transcription factor GABP as a downstream target for the Raf/MEK/ERK signal transduction pathway. Interestingly, Elk-1 and Sap-1 are also reported to be ERK targets (46). The activity of these Ets family transcription factors is regulated by the phosphorylation of a cluster of C-terminal S/T-P motifs, which follow the ERK consensus sequence. Experiments with both activated and dominant negative mutants of MEK-1 and ERK showed that MAPK activity is necessary for activation of Elk-1 and Sap-1 in vivo. GABP α and GABP β contain such potential -P-X-S/T-P- or -S/T-P- phosphorylation sites (Fig. 1C). We are currently mapping the phosphorylation sites in vivo and in vitro, and in future experiments we will study the functional significance of these sites in terms of integration of signals from distinct but interconnected pathways (Fig. 7). Specifically, the role of stress-related pathways (10, 14, 17, 31), for example, JNK/SAPK- and p38/RK-activating cascades, for modulation of GABP activity on the HIV-1 promoter will be evaluated.

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