The GABP-responsive Element of the Interleukin-2 Enhancer Is Regulated by JNK/SAPK-activating Pathways in T Lymphocytes*

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T cell activation leads via multiple intracellular signaling pathways to rapid induction of interleukin-2 (IL-2) expression, which can be mimicked by costimulation with 12-O-tetradecanoylphorbol-13-acetate (TPA) and ionomycin. We have identified a distal IL-2 enhancer regulated by the Raf-MEK-ERK signaling pathway, which can be induced by TPA/ionomycin treatment. It contains a dyad symmetry element (DSE) controlled by the Ets-like transcription factor GA-binding protein (GABP), a target of activated ERK. TPA/ionomycin treatment of T cells stimulates both mitogen-activated ERK, as well as the stress-activated mitogen-activated protein kinase family members JNK/SAPK and p38. In this study, we investigated the contribution of the stress-activated pathways to the induction of the distal IL-2 enhancer. We show that JNK- but not p38-activating pathways regulate the DSE activity. Furthermore, the JNK/SAPK signaling pathway cooperating with the Raf-MEK-ERK cascade in TPA/ionomycin-induced DSE activity.

T cell activation requires at least two signals mediated by T cell receptor-CD3 complex and a costimulatory signal such as ligand binding to CD28 (reviewed by Cantrell (1)). This co-stimulation can be mimicked by phorbol ester and calcium ionophore and results in rapid increase of free intracellular calcium ions, activation of mitogen-activated protein kinase (MAPK)1 cascades, and subsequent induction of IL-2 expression (1). Several lines of evidence support the hypothesis that activation of MAPK family members ERK (extracellular signal-regulated kinase) and JNK (c-Jun N-terminal kinase) play an important role in T cell activation and IL-2 expression (2–8).

The ERK-activating pathway also designated Raf-MEK-ERK cascade involves Raf activation at the plasma membrane, leading to subsequent phosphorylation and activation of the dual specificity kinase MEK (MAPK/ERK kinase), which in turn activates ERK (9, 10). JNK/SAPK (stress-activated protein kinase) (11, 12) is triggered by MKK7 (MAP kinase kinase 7) (13, 14) or SEK/MKK4 (SAPK-ERK kinase) (15, 16). Multiple upstream activators of SEK have been described, including SPRK/MLK3 (SH3-domain-containing proline-rich kinase/mixed-lineage kinase 3) (17). JNK activation in T cells presumably contributes to proliferation (2), whereas in other cell types it may be involved in differentiation (18) or proapoptotic processes (19). Another signaling cascade leads to activation of a third member of the MAPK family, p38 (20). MKK6 (MAP kinase kinase 6) has been identified as the physiological p38 activator (21). JNK- and p38-activating pathways are strongly triggered by inflammatory cytokines (TNF-α, IL-1), UV radiation, and chemical stress inducers such as arsenite and anisomycin, indicating a role in cellular stress response (22). However, a recent report describes an involvement of p38 in IL-2- and IL-7-induced T cell proliferation (23).

For each MAPK, many substrates have been identified including kinases such as MAPKAPK-2, -3pK, and diverse transcription factors (22) mediating signals of kinase cascades into changed gene expression. Several members of the Ets family of transcription factors have been characterized as targets for MAPKs, including Elk-1 (24–26), Sap-1a and Sap-2 (27, 28), PEA3 (29), ERM (30), ER81 (31), ERF (32), Ets1 (33), and Yan and Pointed-P2 (34). In addition, the Ets-related transcription factor, GA-binding protein (GABP), is phosphorylated by ERK controlling the Raf-responsive element of the HIV-1 promoter (35). GABP, consisting of two subunits, was originally identified as a protein complex that binds to a purine-rich hexanucleotide 5′-CGGAAR-3′ within the ICP4 promoter of Herpes simplex virus 1 (36, 37).

Recently, we have identified another GABP response element within a distal IL-2 enhancer and observed increased IL-2 promoter/enhancer activity when GABP was overexpressed (38). The IL-2 gene transcription is tightly regulated...
and occurs only when T cells are activated via the TCR complex and a costimulatory signal (39). The IL-2 promoter/enhancer contains several binding sites for transcription factors regulated by multiple signaling events. The combined NFAT-AP1 sites in the 320-base pair minimal promoter/enhancer region play a crucial role in the integration of different signaling cascades (39). However, upstream DNA sequences from position −321 to approximately −600 of the IL-2 gene that are highly conserved between mouse and man are shown to enhance the promoter activity (40). We have identified an inducible enhancer element spanning the region from position −502 to −413 relative to the transcriptional start site. Within the distal enhancer, the GABP binding site consists of two palindromic Ets-related elements (ERE) and were designated dyad symmetry element (DSE) (38). We observed a potentiated transcriptional activity of the DSE after costimulation with 12-O-tetradecanoylphorbol-13-acetate (TPA) and ionomycin (TPA/ ionomycin) of T cells as well as by overexpression of Ras, c-Raf, and ERK in combination with TPA stimulation (38). As TPA/ ionomycin is known to also activate stress-activated protein kinase JNK/SAPK in T cells (2), we investigated whether stress-activated MAPKs contribute to DSE activity in T cells.

Here, we report that overexpression of SPRK/MLK3, an activator of SEK-JNK-SAPK cascade, strongly induces DSE activity. The SEK-SAPK cascade partially mediates induced DSE-dependent transcription, and it cooperates with the Raf-MEK-ERK cascade in TPA/ionomycin-induced DSE activity. The DSE activity is strongly dependent on the Ets-core motifs, which are bound by GABP. Both subunits of GABP are phosphorylated upon JNK activation in vivo and three different isoforms of JNK/SAPK in vitro. These data suggest that JNK/SAPK and ERK activation converge on GABP to regulate DSE activity.

MATERIALS AND METHODS

Cell Lines and Antibodies—A3.01 human T lymphoma cells were grown in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS) to a density of 8 × 10⁶ cells/ml. The human embryonic kidney cell line HEK293 and murine fibroblast cell line NIH-3T3 were cultured in DME medium supplemented with 10% FCS. Cells were incubated at 37 °C in humidified air with 5% CO₂. Antibodies raised against ERK2 (sc-154), JNK1 (sc-474), p38 (sc-535), and Flag-tag (sc-807) were purchased from Santa Cruz Biotechnology, Inc. Antibodies against GABP-α, GABP-β, and GST were obtained from rabbits immunized with correspondingly bacterially expressed and purified proteins. The monoclonal antibodies against HA-tag (12CA5) were produced and purified as described previously (38). A3.01 cells were split 4 × 10⁶ cells/ml 1 day before transfection. A DRMIRE™-C-based transfection protocol was used according to manufacturer’s instructions (Life Technologies, Inc.). Cells were seeded in 6-well plates 7 × 10⁵ cells/well in 1.5 ml of Opti-MEM (Life Technologies, Inc.) containing 3 μl of DMRIE and up to 3 μg of vector DNA. Transfections for luciferase assays were performed with 0.6 μg of reporter construct plus 2 μg of plasmid containing various cDNAs. Unless otherwise indicated, 24 h after transfection, cells of each well were harvested in 100 μl lysis buffer (50 mM Na-MES, pH 7.8, 50 mM Tris-HCl, pH 7.8, 10 mM dithiothreitol, 2% Triton X-100). The crude cell lysates were cleared by centrifugation, and 50 μl of precleared cell extracts were added to 50 μl of luciferase assay buffer (125 mM Na-MES, pH 7.8, 125 mM Tris-HCl, pH 7.8, 25 mM Mg-acetate, 2 mg/ml ATP). Immediately after injection of 50 μl of 1 mM Nα-μcifin (Applichem) into each sample, the luminescence was measured for 5 s in a luminometer (Berthold). The luciferase activities were normalized on the basis of β-galactosidase activity of cotransfected RSV-β-gal vector and protein content. The β-galactosidase assay was performed with 20 μl of precleared cell lysate according to a standard protocol (47). Mean and standard deviations of at least three independent experiments each done twice or triplicates are shown in the figures.

A3.01 cells were stimulated with 10 ng/ml TPA or 0.5 μM ionomycin (Sigma) for 24 h unless otherwise indicated. The MEK-specific inhibitor, PD098059 (Calbiochem), was used in a 30 μM concentration of a 15 μM stock solution in Me2SO. The p38-specific inhibitor, SB203580 (Calbiochem), was used at a concentration of 2 μM of a 20 μM stock solution in Me2SO. Cells were preincubated with either inhibitor 20 min before stimulation.

Immunoprecipitation, Kinase Assay, and Immunoblotting—Cells were lysed in RIPA buffer (described above), and cell debris was removed by centrifugation. Supernatants were incubated with different anti sera for 2 h at 4 °C. The immune complexes were precipitated with protein-A agarose (Boehringer) and washed twice with high-salt RIPA buffer containing 0.1% SDS. Immunoblots were analyzed by an image analysis system (Amersham, Little Chalfont, United Kingdom).

Purification of Bacterially Expressed Proteins—Bacterially expressed proteins GABP-α, GABP-β, 3pK-K(M), GST-c-Jun(1–135), and GST-SAPK were purified as described earlier (35).

Electromobility Shift Assays (EMSAs)—Crude nuclear extracts of A3.01 cells were prepared as described previously. 2 μg of nuclear protein from various amounts of preincubated GABP proteins were preincubated on ice with 2 μg of poly(dI-dC) (Boehringer) and 1 μg of bovine serum albumin in bandshift buffer (60 mM Hepes, pH 7.9, 3 mM dithiothreitol, 3 mM EDTA, 150 mM KCl, 12% Ficoll). After 10 min, 12.5 fmol of a 32P-labeled oligonucleotide (equivalent to approximately 50,000 cpm) was added in a total volume of 10 μl, incubated at room temperature for 15 min, and loaded onto 5% native polyacrylamide gels.
in 0.4 X Tris borate-EDTA buffer. Upon fractionation, gels were dried and exposed for autoradiography. The following oligonucleotides were used as labeled probes and unlabeled competitors, which were optionally added to the DNA-protein-binding reaction: DSE (wild type), 5'-TCTGAAAACGGAAAACCTTACCTTTTGTTTTAATC-3'; DSE-Am, 5'-TCTGAAAACGGAAAACCTTACCTTTTGTTTTAATC-3'; ERE-A (wild type), 5'-AATACCTGGTGTTTTAATC-3'; ERE-Bm, 5'-TCTGAAAACGGAAAACCTTACCTTTTGTTTTAATC-3'; ICP4 (GABP site), 5'-AGCTTGGCGAACGGGACGGCAGCCCGCAGCCATGTC-3'.

For supershift EMSAs, 2 μg of purified preimmune serum or anti-GABP or anti-GAPB-γ were incubated on ice with 2 μg of nuclear extracts for 20 min before adding the labeled DSE oligonucleotides. The DNA-protein complexes were separated on 4% native polyacrylamide gels.

RESULTS

SPRK/MLK3 Strongly Induces the Activity of the DSE of the Distal IL-2 Enhancer—Overexpression of SPRK/MLK3 activates JNK/SAPK and p38 via SEK and MKK6, respectively, in COS1 and HEK-293 cells (17). We investigated the effects of these stress pathways on the DSE activity in the CD4 positive T cell line A3.01 using a four-copy DSE cloned in front of a minimal thymidine kinase (tk) promoter construct in transient transfection assays. Compared with vector control, the DSE activity increased 28-fold when SPRK/MLK3 was cotransfected (Fig. 1A). As a positive control, we stimulated A3.01 cells with TPA/ιonomycin, which lead to IL-2 secretion (data not shown) and resulted in a 16-fold induction of the DSE activity (Fig. 1A). Mutations of the GGAA-Ets-core motifs of the DSE abolished activity (Fig. 1A), which could not be further enhanced by ionomycin (Fig. 2A, upper panel). JNK1 was only 5-fold activated by TPA; ionomycin alone had no activating effect. Co- stimulation with TPA/ionomycin led to a strong synergistic activation (Fig. 2A, middle panel). Interestingly, p38 showed a similar pattern of regulation as JNK, as it was only modestly activated by TPA and synergistically by TPA plus ionomycin (Fig. 2A, lower panel). Thus, all three MAPKs are strongly activated under TPA/ionomycin costimulation.

To dissect the contribution of each MAPK pathway to the regulation of the DSE, we tested constitutively active kinases to activate ERK, JNK/SAPK, and p38 in A3.01 cells. MKK6 (EE), which has the two activating phosphorylation sites replaced by glutamic acid residues rendering it to a constitutively active kinase (21), stimulates p38 activity in A3.01 cells (Fig. 2B, lane 5). In contrast to the data of Tibbles et al. (17) observed in COS1 and HEK-293 cells, overexpression of SPRK/MLK3 in A3.01 cells only activates JNK/SAPK and has no effect on p38 or ERK activity (Fig. 2B, lane 4). The kinase domain of Raf (Raf-BXB) has been shown to be constitutively active in diverse cell lines such as NIH-3T3, HEK-293, and COS cells. However, it is only slightly active in T cells but can be strongly activated upon TPA stimulation (3, 43).2 We showed previously that Raf-BXB in combination with TPA induces DSE activity (38). In this study, we used a membrane-targeted version of Raf-BXB by fusing it to the CAAX motif of Ki-Ras (Raf-BXB-CX). Raf-BXB-CX expression was sufficient to induce ERK activity to an extent comparable with that induced by TPA/ionomycin without affecting JNK/SAPK or p38 activity (Fig. 2B, lane 3).

After establishing selective MAPK activation in A3.01 cells, we performed transient transfection assays using the 4xDSE-tk luciferase construct to test individual effects on DSE activity. Transfection of increased amounts of either Raf-BXB-CX or SPRK/MLK3 expression vectors led to dose-dependent strong induction of DSE activity of up to 16- and 30-fold, respectively (Fig. 3A). Surprisingly, overexpression of constitutively active MKK6 (MKK6 (EE)) resulted in only marginal effects on DSE activity (Fig. 3A), whereas it significantly activated p38 (Fig. 2B). The tk-minimal promoter alone was not

2 Flory, E., Weber, C. K., Chen, P., Hoffmeyer, A., Jassoy, C., and Rapp, U. R. (1998) J. Virol., in press.
significantly responsive to any of the activating kinases (Fig. 3A). Moreover, point mutations in the Ets-core consensus sequences abolished Raf-BXB-CX and SPRK/MLK3 responsiveness of the DSE in transient transfection experiments (Fig. 3A).

Taken together, both active Raf and SPRK/MLK3 up-regulate DSE activity, whereas active MKK6 is not sufficient to trigger DSE-dependent transcription.

**Block of the ERK and JNK/SAPK Pathway Abolishes the TPA/Ionomycin-induced DSE Activity**—Because TPA/ionomycin-induced activation of ERK, JNK, and p38 correlated with strong DSE activity, we addressed the question whether these MAPKs play a role in the induction of DSE activity. Therefore, cotransfections were performed using the 4xDSE-tk luciferase construct together with interfering kinase mutants of MKK6, SEK, JNK/SAPK, and ERK. Kinase-inactive MKK6 (MKK6 (A)) showed no inhibitory effect on TPA/ionomycin-induced DSE activity (Fig. 3B). Also, pretreatment with the p38-specific inhibitor, SB203580 (49), had no effect on TPA/ionomycin DSE induction. The concentration used was sufficient to almost abolish p38 activity without affecting ERK or JNK activities (data not shown).

Interfering mutants of both, SEK (SEK(K-R)) and SAPK (SAPKβ (K-R)) partially inhibited the TPA/ionomycin effect on DSE (Fig. 3B). Moreover, two different interfering mutants of ERK2 (ERK2 C3 and B3) only partially inhibited the DSE induction (Fig. 3B). Using the MEK-specific inhibitor, PD098059 (50), an inhibition of 65% of the TPA/ionomycin-induced DSE activity is demonstrated (Fig. 3B). The concentration of the inhibitor used was sufficient to abolish catalytic activity of ERK without affecting JNK or p38 activity (data not shown). In T cells transfected with interfering SAPKβ (K-R) preincubated with MEK inhibitor, PD098059, the TPA/ionomycin-induced DSE activity was almost abolished (Fig. 3B).

These data show that both Raf-MEK-ERK and SEK-JNK/ SAPK pathways mediate TPA/ionomycin-induced DSE activity in a cooperative fashion. However, the MKK6-p38 pathway seems to have no effect.

**GABP Is the Predominant Binding Factor of the DSE in A3.01 T Cells**—The involvement of different pathways on DSE activity suggests a complex regulation of this element. Previously, we have described GABP as one DSE binding factor in Jurkat nuclear extracts (38). To elucidate the effects of T cell stimulation on DSE binding factors, we characterized the binding factors of A3.01 T cells. We performed EMSAs using labeled DSE oligonucleotides in reconstitution experiments with recombinant GABP proteins (Fig. 4A), competition assays (Fig. 4B), and supershift analysis (Fig. 4C).

Nuclear extracts of stimulated A3.01 cells were incubated with radiolabeled DSE oligonucleotides and then separated on a native polyacrylamide gel. Independent of stimulation, three major complexes were observed, referred to as complexes I, II, and III (Fig. 4A, lanes 15–18), whereby complex II appeared in high resolution as two bands (Iia and Iib). Recombinant, purified GABP-α and GABP-β showed a very similar migratory behavior when incubated with DSE oligonucleotides compared with crude nuclear extracts (Fig. 4A, lanes 14–18). To elucidate the composition of the complexes, we used GABP-α and -β subunits alone or in combination with either wild type DSE or DSE oligonucleotides containing a mutation in one of the two Ets-related elements (DSE-Am). Recombinant GABP-α, which contains the Ets domain required for DNA contact (37), binds to DSE-Am as a single shifted complex, corresponding to complex III (Fig. 4A, lanes 1–4). GABP-α alone does not bind (lane 5), but addition of GABP-α resulted in a slower migrating complex. This heterodimeric formation of both GABP subunits corresponds to complex II (Fig. 4A, lanes 6–9). Binding assays using wild type DSE oligonucleotides with GABP-β together with increasing amounts of GABP-α resulted in a heterodimeric GABP formation at lower concentrations of α-subunit (corresponding to complex II, lanes 10 and 11). The two complexes Iia and Iib of nuclear extracts might be explained by the existence of different GABP-β isoforms (51, 52).

At higher concentrations of GABP-α, another slower migrating complex was observed, most likely a tetrameric association of two heterodimeric GABP proteins bound to DSE (lanes 12–14), which is also observed with other promoter elements and seems to be critical for strong transcriptional activation (37, 53,
was used at a concentration of 30 μM to inhibit kinase activity in unstimulated cells (data not shown). PD098059 was added 20 min before stimulation. Both inhibitors had no effect on DSE activity in control transfection cells were left untreated or stimulated with TPA/ionomycin or corresponding background vector (data not shown). Overexpression of constitutively active c-Raf (Raf-BXB-CX) or SPRK/MLK3, but not constitutively active MKK6 (EE), is sufficient to activate DSE-dependent transcription, for which the GABP binding sites are required. A, A3.01 cells were cotransfected with 4xDSE-tk luciferase construct (4xDSEx-tk) together with either increasing amounts (0.5, 1.0, or 2.0 μg of vector DNA per transfection) of Raf-BXB-CX, SPRK/MLK3, or MKK6 (EE) expression vector or corresponding empty expression vector pRSVα (vector). As a control, 2.0 μg of background vector (vector) or either expression vector together with tk-luciferase construct (tk) were cotransfected. To test requirement of the Ets-consensus sequence, the 4xDSE/mut-tk luciferase construct with mutations in both Ets-core motifs were cotransfected with 2.0 μg of background vector (vector) or either expression vector. 24-h post-transfection cells were harvested, and luciferase assays were performed (see above). Relative luciferase activities of cells transfected with each cDNA expression vector are based on activities of cells transfected with the same amount of empty expression vector (pRSVα). Efficiency of MKK6 (EE) expression in terms of activating transcriptional activity was determined on an HIV-1 long terminal repeat-driven reporter construct (data not shown). B, kinase-inactive versions of ERK2, SEK1, SAPKβ, and MKK6 impair TPA/ionomycin-induced DSE-driven reporter expression. A3.01 cells were cotransfected with 4xDSE-tk luciferase construct together with diverse kinase inactive mutants of ERK2 (B3 and C3), SEK1 (KR), SAPKβ (KR), MKK6 (A), or corresponding background vector (vector) as indicated. 18-h post-transfection cells were left untreated or stimulated with TPA/ionomycin for 24 h before harvest. PD098059 (PD) or SB203580 (SB) were added 20 min before stimulation. Both inhibitors had no effect on DSE activity or kinase activity in unstimulated cells (data not shown). PD098059 was used at a concentration of 30 μM, which blocked ERK activation to 95% without affecting JNK or p38 activity, and SB203580 at a concentration of 2 μM, which inhibited p38 activity to 85% without any influence on ERK or JNK activity (data not shown). Efficient inhibition of p38 activity by MKK6 (A) was determined by p38-specific immune complex kinase assay described above (data not shown). No specific transcriptional inhibition by dominant negative kinase mutants was observed when judged by cotransfection with an RSV-driven reporter construct (R5β-gal) and subsequent β-galactosidase assay (data not shown). Luciferase assays were performed as described under “Materials and Methods.” Relative luciferase activity of stimulated cells is based on unstimulated cells equally transfected.

54). This complex of purified GABP proteins shows the same migratory behavior as complex I of nuclear extracts, indicating that complex I consists only of tetrameric GABP complex. Using mutant DSE-Am oligonucleotides, which allows GABP binding only at one Ets-binding motif, the tetrameric formation of GABP on the DSE can be prohibited. When crude nuclear extracts were incubated with these oligonucleotides, no complex I was detected (data not shown).

To confirm that the complexes contain GABP, we used an oligonucleotide as competitor containing the GABP-specific binding sites of the ICP-4 promoter (Fig. 4B, lanes 11–13), which was used for the identification of GABP (36). This oligonucleotide competed as efficiently as wild type Ets-related element A (ERE-A) for DSE-bound protein complexes (Fig. 4B, lanes 2–4), whereas mutations in the Ets-core motif in either ERE-A or ERE-B oligonucleotides abolished their ability to compete with labeled DSE (Fig. 4B, lanes 5–10). Finally, incubation of 3A.01 nuclear extracts with specific anti-GABP-α or anti-GABP-β antibodies supershifted the complexes of 3A.01 nuclear extracts (Fig. 4C, lanes 3 and 4, indicated by ss).

These results suggest that GABP is the predominant DSE binding factor, and complex I, which seemed to be responsible for the transcriptional activity (38), was characterized as the tetrameric GABP complex. Interestingly, the DNA binding activity of GABP seems not to be regulated by stimulation with TPA, ionomycin, or their combination, as no significant changes in DNA binding activity were observed when nuclear extracts of unstimulated or stimulated A3.01 cells were used for EMSA (Fig. 4A, lanes 15–18).

GABP Is Phosphorylated upon JNK/SAPK Stimulation in Vivo and by JNK/SAPK in Vitro—Because GABP is the predominant DSE-binding factor and GABP binding sites are necessary for transactivation by Raf and SPBK/MLK3, we tested whether GABP is a direct substrate not only for mitogen-stimulated ERK (35, 55) but also for other MAPK family members. We have shown that GABP factors are also phosphorylated in vivo upon stimulation of HEK-293 cells with TPA and serum (35). To study the effects of SAPK on phosphorylation of GABP in vivo, this cell line was transfected with GABP-α and -β expression vectors alone or in combination with SAPKβ expression vector and metabolically labeled with [32P]orthophosphate. Cells were treated with anisomycin to strongly activate SAPK without affecting ERK activity. Fig. 5 shows the autoradiography (Fig. 5A) and corresponding immunoblot (Fig. 5B) of immune precipitated GABP subunits. We observed an increased phosphorylation of both GABP subunits upon anisomycin stimulation, which was further increased when SAPKβ was overexpressed, implicating a function in GABP phosphorylation. Treatment of HEK-293 cells with anisomycin leads to SEK, JNK/SAPK, and p3K activation as well as to p38 phosphorylation (Fig. 44).

We next tested the ability of these kinases to phosphorylate GABP in vivo, using ERK as a positive control. In vivo activated and immunopurified GST-tagged SAPKβ but not Flag-tagged p38 phosphorylated both subunits of GABP (Fig. 6B). Bacterially expressed, purified, and preactivated GST-SAPKαβ also was able to phosphorylate both GABP subunits in vitro like GST-c-Jun (Fig. 6C). Both activated SEK and p3k did not phosphorylate GABP (data not shown).

To extend the phosphorylation studies to another JNK/SAPK isozyme, JNK1/SAPKαβ, in addition to p38 and ERK of untreated or TPA/ionomycin-stimulated A3.01 cells, was tested for their ability to phosphorylate GABP factors in vitro (Fig. 6A). Consistent with the above described data of the in vitro kinase assays and with our transactivation data, ERK and JNK1 but not p38 functioned as a GABP kinase (Fig. 6A).

The ability of three different isoforms of JNK/SAPK (SAPKα, -β, and JNK1) to phosphorylate GABP in vivo, in combination with the in vivo phosphorylation of GABP upon SAPK activa-
It is worth noting that GABP- shows corresponding immunoblot using anti-GABP-

GABP-A3.01 T cells (w/o then in Panel C DSE oligonucleotide. Whereas wild type DSE contains 2 EREs, one of both is mutated in ERE-A mutated DSE. Different amounts indicated in the figure in nanograms transfected in HEK-293 cells. 48 h later, cells were subjected to phosphatase-free DMEM 2 h before addition of [32P]orthophosphate. 1 h later, cells were stimulated with anisomycin for 1 h or left untreated as indicated in the figure. Cells were lysed in RIPA buffer. Lysates were split into 2 halves, one of which was incubated with anti-GABP- (lanes 1–4), the other half with anti-GABP- antisera (lanes 5–8). Immune complexes were precipitated with protein-A agarose, washed, subjected to SDS-polyacrylamide gel electrophoresis, and electrobotted on polyvinylidene difluoride membrane. Panel A shows autoradiography; panel B shows corresponding immunoblot using anti-GABP- and - antisera. It is worth noting that GABP- protein load in lane 4 is four times less then in lanes 1–3.

FIG. 4. GABP- are the predominant DSE binding factors of A3.01 nuclear extracts. A, either 2 μg of nuclear extracts of 4-h stimulated A3.01 T cells (w/o, without stimuli, lane 18; T, TPA, lane 16; I, ionomycin, lane 17; and T+I, TPA/ionomycin, lane 18) or recombinant GABP proteins (lanes 1–14) were incubated with radiolabeled wild type DSE (lanes 10–18) or ERE-A mutated DSE (DSE-Am) (lanes 1–9) oligonucleotides. Whereas wild type DSE contains 2 EREs, one of both is mutated in ERE-A mutated DSE. Different amounts indicated in the figure in nanograms of recombinant GABP- (α) and GABP- (β) alone or in combinations were used for EMSA (lanes 1–14). B, for competition assays, 10-, 50-, or 250-fold molar excess of unlabeled ERE-A (lanes 2–4), mutant ERE-A (ERE-Am, lanes 5–7), mutant ERE-B (ERE-Bm, lanes 8–10), or ICP4 oligonucleotide containing GABP binding sites (ICP4, lanes 11–13) were added to the binding reaction of 2 μg of A3.01 nuclear extracts and labeled DSE oligonucleotide. Panel C shows supershift experiments with anti-GABP-specific antiserum. 2 μg of nuclear A3.01 extracts were incubated with preimmune serum (PI, lane 2), anti-GABP- serum (α, lane 3), anti-GABP- serum (β, lane 4), or without antibodies (−, lane 1) for 15 min before radiolabeled DSE oligonucleotides were added. To separate supershifted complexes, a 4% polyacrylamide gel was used. EMSAs were performed as described under “Materials and Methods.” The autoradiographies display three major complexes (I, II, III), supershifted complexes (ss), and unbound oligonucleotides (free probe, fp).

FIG. 5. Both GABP subunits are phosphorylated in vivo. GABP- and - expression vectors alone (lanes 1, 2, 5, and 6) or in combination with pEG-SAPKβ (lanes 3, 4, 7, and 8) were transiently transfected in HEK-293 cells. 48 h later, cells were subjected to phosphatase-free DMEM 2 h before addition of [32P]orthophosphate. 1 h later, cells were stimulated with anisomycin for 1 h or left untreated as indicated in the figure. Cells were lysed in RIPA buffer. Lysates were split into 2 halves, one of which was incubated with anti-GABP- (lanes 1–4), the other half with anti-GABP- antisera (lanes 5–8). Immune complexes were precipitated with protein-A agarose, washed, subjected to SDS-polyacrylamide gel electrophoresis, and electrobotted on polyvinylidene difluoride membrane. Panel A shows autoradiography; panel B shows corresponding immunoblot using anti-GABP- and - antisera. It is worth noting that GABP- protein load in lane 4 is four times less then in lanes 1–3.

DISCUSSION

We characterized the Ets-core motifs of a distal IL-2 enhancer as a SPRK/MLK3-SEK-JNK/SAPK responsive element. In the TPA/ionomycin induction of the DSE activity, the JNK/SAPK activating pathway cooperates with the Raf-MEK-ERK signaling pathway. Block of both cascades almost abolished the induction, whereas activation of either of these signaling cascades is sufficient to induce DSE activity independently. Despite being activated, we observed no critical contribution of the MKK6-p38 pathway in DSE regulation. The DSE activity is strongly dependent on the Ets-core motifs, which are bound by GABP. Both subunits of GABP are phosphorylated upon JNK activation in vivo and three different isoforms of JNK/SAPK, but not p38, in vitro. These data suggest that ERK and JNK activation converge on GABP to regulate DSE activity, which enhances IL-2 induction (Fig. 7).

In our experimental study, we used SPRK/MLK3 as a JNK/SAPK activator. SPRK/MLK3 overexpression induced the IL-2 promoter/enhancer activity in A3.01 T cells (data not shown) and is a strong transactivator of the DSE. Interestingly, the SPRK/MLK3-induced DSE activity exceeded the induction by TPA/ionomycin (Fig. 1). Because SPRK/MLK3 activates SAPK to the same extent as TPA/ionomycin after 20 min of stimulation (Fig. 2), the difference might be explained by the duration of the signal. JNK/SAPK activity is triggered by TPA/ionomycin maximally after 20 min and returns to baseline activity after 2 h (data not shown). In contrast, SPRK/MLK3 overexpression leads to sustained high JNK activity (Fig. 2).

Employing a constitutively active kinase version of MKK6, we were not able to measure significant induction of DSE activity. In addition, neither an interfering kinase mutant of MKK6 nor the specific p38 inhibitor, SB203580, impaired the TPA/ionomycin-induced DSE activity. Although both JNK and p38 are stimulated by an overlapping spectrum of stimuli, activation of these kinases shows specific cellular responses. The specificity might be a consequence of the selective choice of the transcription factor mediating the kinase activity. We identified GABP as a target of JNK/SAPK, but not p38. C-Jun is another factor that is activated by JNK, which also has not yet been demonstrated to be phosphorylated by p38. Even though both JNK and p38 phosphorylate Elk, both kinases show different preferences for the phospho-acceptor sites (28). The elucidation of effectors, which are either targeted by JNK or p38, will provide further insights into the specificities of both pathways.
visualized by autoradiography. Equal substrate content was determined in immunoblots (not shown).

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cooperates in the induction of transcriptional activity via the GABP-

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activation. ERK and JNK and transmits the signal into potentiated transcriptional

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pathways. ERK and JNK activation of the distal IL-2 enhancer.

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activation. T cell activation leads to rapid induction of GABP, as both subunits of GABP contain multiple potential MAPK

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affinity (32). In case of the GABP-responsive element, cooperation of diverse MAPKs has been identified as a target for ERK, in which ERK phosphorylates GABP, as the DSE binding complexes of A3.01 nuclear extracts have been identified as monomeric, dimeric, and tetrameric GABP complexes (Fig. 4). Interestingly, the transcription factor is a target of two diverse MAPKs. However, other Ets family members such as Sap-1a (27) and PEA3 (29) have been described as substrates for mitogenic-induced ERK and stress-activated JNK/SAPKs. This implies that signal convergence is an important mechanism in regulation of these transcription factors. The regulation of GABP by these kinase cascades is based on an as yet unidentified mechanism, as T cell stimulation neither modifies the subcellular localization of GABP subunits (not shown) nor dramatically changes the DSE binding affinity (32).

The most prominent candidate for linking phosphorylation cascades to transcriptional activity is the transcription factor GABP, as the DSE binding complexes of A3.01 nuclear extracts have been identified as monomeric, dimeric, and tetrameric GABP complexes (Fig. 4). Interestingly, the transcription factor is a target of two diverse MAPKs. However, other Ets family members such as Sap-1a (27) and PEA3 (29) have been described as substrates for mitogenic-induced ERK and stress-activated JNK/SAPKs. This implies that signal convergence is an important mechanism in regulation of these transcription factors. The regulation of GABP by these kinase cascades is based on an as yet unidentified mechanism, as T cell stimulation neither modifies the subcellular localization of GABP subunits (not shown) nor dramatically changes the DSE binding affinity (32).

Because GABP is ubiquitously expressed, we tested whether the regulation of DSE activity occurs also in non-T cell lines. Indeed, in the human embryonic kidney cell line HEK-293, the DSE activity also is up-regulated by constitutively active Raf and SPRK/MLK3 overexpression (data not shown), and GABP phosphorylation is induced upon TPA treatment in vitro (35), which activates ERK but not JNK/SAPK or p38 (44). In this study, we addressed the question whether GABP phosphoryl-
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ation occurs in vivo, when JNK is active. To exclude the phosphorylation by ERK, we treated HEK-293 cells with the stress inducer anisomycin, which strongly up-regulates JNK/SAPK but does not affect ERK activity (44). Under these conditions, both subunits of GABP are induced phosphorylated and even more when SAPK was overexpressed. Also in NIH-3T3 fibroblasts (35) and pituitary GH4 cells (55), an induction of GABP-responsive elements occurs via extracellular stimuli. In this context, it might be interesting to test whether GABP-responsive promoters of house-keeping genes (54, 56) can be activated beyond the level of constitutive activity by triggering intracellular signaling events. In particular, GABP has been shown to regulate the expression of nuclear-encoded mitochondrial proteins, cytochrome oxidase subunits IV and Vb, and the mitochondrial transcription factor mtTF-1, which is constitutively expressed but can also be regulated in response to changes in the redox state of the cell (for example, by hypoxia). Hypoxia induces ERK, JNK, and p38 (57). This observation, combined with our finding that the GABP-responsive element is regulated by ERK and JNK, allows us to speculate that phospho-
gulation of GABP mediates hypoxia-regulated expression of mitochondrial proteins involved in cellular respiration. Further-
more, several viruses such as herpes simplex virus 1 (36, 58), adenovirus (53), and Moloney murine leukemia virus (48) as well as human immunodeficiency virus 1 (35) recruit GABP for viral gene expression. Indeed, we observed an induced HIV-1 long terminal repeat activity when we overexpressed SPRK/MLK3 in A3.01 T cells. In the light of our observations that GABP is a shared target of ERK and JNK/SAPK, one might speculate that GABP converts diverse signals mediated by ERK and JNK/SAPK into induced gene expression.

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