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Transcriptional Activation and Transformation by FosB Protein Require Phosphorylation of the Carboxyl-Terminal Activation Domain

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The transcription factor AP-1, composed of Fos-Jun dimers, mediates some aspects of the cellular response to growth factors. Transcriptional activation and neoplastic transformation by FosB, a member of the Fos family of proteins, require the presence of a potent C-terminal activation domain. Here we show by mutational analysis that the FosB C-terminal domain has a proline-based motif that is essential for both of these functions. Phosphopeptide mapping experiments show that the C terminus of FosB is phosphorylated within a cluster of functionally redundant serine residues that is adjacent to this proline-based motif. Mutation of these serine residues to alanine severely reduces the ability of this region to function as an activation domain and inhibits the ability of FosB protein to function as a transforming protein. Several observations suggest that the kinase responsible for phosphorylation of these sites is distinct from the mitogen-activation protein kinases and stress-activated protein kinases. Our results show that transcriptional activation and neoplastic transformation by the FosB protein are dependent on phosphorylation within the C terminus. This form of control may provide a potential mechanism of signal integration at the level of a single transcription factor.

Peptide growth factors control the proliferation of many cell types. The response to growth factors is mediated by their specific interactions with and activation of cell surface receptors. The activated receptors initiate a complex series of biochemical events that eventually results in cell division. Among these biochemical events, those that activate transcription factors are important, as classical studies have shown that proliferation in response to growth factors requires the synthesis of new proteins. The transcription factor AP-1 is one receiver of growth factor-generated signals, and accumulating evidence strongly suggests that the activation of AP-1 in response to peptide growth factors is critical for the proliferative response (3, 17, 18, 20, 24).

The transcription factor AP-1 is composed of a mixture of heterodimeric complexes of proteins derived from the Fos and Jun families (for a review, see reference 4). The mechanism of AP-1 activation by growth factors is complex. One major level of control is a large increase in the synthesis of new mRNAs and proteins for some of the AP-1 component subunits in response to growth factors. For at least some of the genes that encode AP-1 subunits, this increase in de novo synthesis is the results of signaling by the mitogen-activated protein (MAP) kinase pathway. Although the mechanism of induction of different AP-1 subunit genes is not understood in all cases, in at least some cases this induction occurs by phosphorylation of the ternary complex factor family of transcription factors, including Elk-1, by the MAP and stress-activated protein (SAP) kinases (10, 11, 15, 25, 36). This same biochemical pathway is activated by many other mitogenic stimuli, including phorbol esters. A second level of regulation is by the phosphorylation of AP-1 component proteins in response to specific signals. This is best documented in the case of phosphorylation of serines 63 and 73 in the c-*jun* transcriptional activation domain by the SAP/JNK kinases, a modification which greatly increases transcriptional activation by c-*jun* without affecting the level of DNA binding activity (9, 21). Thus, AP-1 activity is regulated at many levels.

Peptide growth factors are not sufficient to induce cell division. Many other environmental factors, including the abundance of cell nutrients, the availability of an appropriate extracellular matrix, and the presence of other cells, are monitored. It is not understood in molecular detail how these other parameters are integrated into the cellular decision to divide, but one possible mechanism is by regulating the activity of the factors induced by peptide growth factors. AP-1 is therefore one possible site of integration of environmental signals. The AP-1 subunit component proteins are phosphoproteins, and phosphorylation in response to peptide growth factors, Ras proteins, and cytokine receptors has been shown to regulate transcriptional activation by AP-1 (8, 9, 21).

The *fosB* gene, a member of the *fos* gene family, has been shown by gene targeting experiments in mice to play a critical role in certain behaviors (7). The *fosB* gene encodes two functionally distinct proteins by the production of alternately spliced transcripts. The long protein, FosB, differs from FosB2 by the presence of a unique 101-amino-acid C-terminal extension; both proteins contain the basic region-leucine zipper (bZIP) motif and bind Jun proteins and DNA with similar efficiency. We and others have previously shown that FosB is a more potent activator of transcription and neoplastic transformation than FosB2 (27, 28, 37, 39). Several lines of evidence support the conclusion that this difference in biologic activity is due to the presence of a C-terminal transcriptional activation domain in the unique region of FosB: (i) the C-terminal region of FosB functions as a potent activation domain when fused to a heterologous DNA binding domain derived from the yeast Gal4 protein, (ii) mutations that diminish the ability of this domain to function as an activation domain in the context of a Gal4-FosB fusion protein inhibit transcriptional activation and transformation in the context of the FosB protein, and (iii) fusion of a variety of different activation domains to FosB2 generates novel transforming fusion proteins (37).

Here we show that FosB protein is synthesized as an inactive

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protein. Full transcriptional activation by FosB protein requires phosphorylation within a cluster of serine residues in the carboxyl terminus. The region targeted for phosphorylation is conserved in all Fos family proteins, suggesting that this may be a common mode of regulation of AP-1 activity. The C-terminal phosphorylations do not appear to be carried out by known activators of AP-1, including the MAP and SAP kinases, suggesting the existence novel regulators of AP-1 function. Phosphorylation of the C terminus of FosB protein represents an important mode of control of AP-1 activity and identifies a potential molecular mechanism for the integration of multiple signals by a single transcription factor.

MATERIALS AND METHODS

DNA manipulations. Sequences encoding the FosB C terminus (amino acids 226 to 338) were cloned as an *Eco*RI/*Xba*I fragment into the vector pSG424, which encodes amino acids 1 to 147 of Gal4 (30). Deletion derivatives were generated by PCR; point substitutions were generated by oligonucleotide-directed mutagenesis and are named by the residues that they delete or mutate.

Sequences encoding amino acids 270 to 338 of the FosB C terminus were fused as an *Eco*RI/*Xba*I fragment into the vector pGEX-KG-1 (31a), a derivative of pGEX-KG that contains an altered polylinker with *Eco*RI and *Xba*I sites in the appropriate reading frames. Mutations within fragment of FosB were generated by oligonucleotide-directed mutagenesis.

FosB mutant proteins were generated by oligonucleotide-directed mutagenesis and were cloned as *Bam*HI/*Xho*I fragments into the vector SLX-CMV, a retroviral vector in which FosB expression is directed by the cytomegalovirus immediate-early promoter (31, 37).

All mutations were confirmed by DNA sequencing.

Reporter gene expression assays. 208F, F9, and 293 cells were maintained in Dulbecco's minimal essential medium plus 10% fetal calf serum (FCS). The activity of Gal4-FosB fusion proteins was measured by transient transfection of 208F cells. Transfections used 0.5 μg of pG5B-CAT (19), 0.5 μg of the β-galactosidase expression plasmid pCH110 (Pharmacia) as a control for transfection efficiency, and 1.5 μg of pSG-FosB plasmids. Six-centimeter-diameter dishes of 208F cells were transfected by using 12 μl of Lipofectamine (Gibco) per dish; the DNA-Lipofectamine mixture was left on cells for 6 h. The cells were incubated for an additional 40 h, and chloramphenicol acetyltransferase (CAT) activity was analyzed as previously described (13). Conversion of chloramphenicol was quantitated by a PhosphoImager; values represent the averages of at least four separate determinations; individual determinations differed by less than 20%. To measure the response of Gal4-FosB to activation of the ERK and SAP kinase pathways, cells were transfected as described above with plasmids expressing Gal4-FosB or Gal4-Elk1(307-428). After transfection, the cells were shifted into medium containing 0.5% FCS; 36 h later, the cells were treated for 8 h with 15% FCS, epidermal growth factor (20 ng/ml), basic fibroblast growth factor (60 ng/ml), phorbol myristate acetate (100 ng/ml), UV light (40 J/m^2, 254 nm), tumor necrosis factor alpha (TNF-α) (50 ng/ml), or cycloheximide (10 μg/ml), for 2 h followed by washout and incubation for an additional 6 h in serum-free medium. CAT activity was then measured as described above.

The expression of different Gal4-FosB fusion proteins was monitored by transient transfection of human 293 cells. Six-centimeter-diameter dishes of 293 cells were transfected with 2.5 μg of the appropriate Gal4-FosB expression plasmid. Forty hours after transfection, nuclear extracts were prepared by previously described methods (2). The expression was determined by immunoblotting using anti-Gal4 DNA binding domain antibody (Santa Cruz Biotechnology).

The activity of FosB proteins was measured by transient transfection of F9 cells. Transfections used 0.5 μg of a reporter construct, pT3B-CAT (in which three copies of the human collagenase AP-1 sequence were placed upstream of the E1b basal promoter [36a]), 0.02 μg of SLX-CMV-c-Jun plasmid, 0.5 μg of SLX-CMV-FosB plasmid, 0.5 μg of pCH110 as a control for transfection efficiency, and 1.0 μg of SK^- (Stratagene). Six-centimeter-diameter dishes of F9 cells were transfected by using Lipofectamine (12 μl/dish); the DNA-Lipofectamine mixture was left on cells for 6 h. The cells were incubated for an additional 40 h, and then CAT activity was analyzed as described above. The values represent the averages of five separate determinations.

In vivo labelling and mapping of FosB proteins. Ten-centimeter-diameter dishes of human 293 cells were transfected with 7.5 μg of SLX-CMV-FosB (or the appropriate mutant), using 30 μl of Lipofectamine per dish. The cells were allowed to grow for an additional 40 h and were then labelled for 4 h with [^{32}P]orthophosphate (1 mCi/ml) in phosphate-free Dulbecco's minimal essential medium. The cells were lysed in radioimmunoprecipitation assay (RIPA) buffer (20 mM Tris [pH 7.5], 150 mM NaCl, 2 mM $MgCl_2$, 0.5% Nonidet P-40) supplemented with 0.5% sodium dodecyl sulfate (SDS), 20 mM β-glycerolphosphate, and 0.1 mM Na_3VO_4. The lysate was passed through a 25-gauge needle five times, incubated at 95°C for 5 min, and cleared by centrifugation in a microcentrifuge. The cleared lysate was diluted in RIPA buffer to give a final concentration of 0.1% SDS, and immunoprecipitations were carried out with affinity-purified antibodies directed against an amino-terminal domain of FosB and protein A-Sepharose. The immunoprecipitates were washed with RIPA buffer plus 0.1% SDS, boiled in Laemmli sample buffer, and separated by SDS-polyacrylamide gel electrophoresis (PAGE). The proteins were then transferred by blotting to Immobilon membranes and digested off the membrane with chymotrypsin. The cleaved peptides were washed and resolved in two dimensions. Electrophoresis was carried out in pH 8.9 buffer at 1,000 V for 25 min, and chromatography was carried out in phosphochromatography buffer (6).

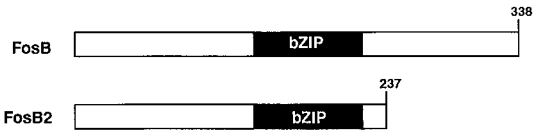

FIG. 1. Schematic diagram of the proteins derived from the FosB gene by alternate splicing. The FosB protein contains a unique 101-amino-acid C-terminal extension compared to the FosB2 protein. Both proteins contain the bZIP motif and bind DNA as heterodimers with Jun proteins with equal efficiency, but the FosB protein is a more potent transcriptional activator and transforming protein.

Preparation of cell extracts, in vitro labelling, and mapping of GST-FosB proteins. Glutathione *S*-transferase (GST)–FosB fusion proteins were purified and bound to glutathione-agarose beads as previously described (34). 208F cells were washed twice with phosphate-buffered saline and then lysed in whole-cell extract buffer (25 mM HEPES [pH 7.6], 300 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.1% Triton X-100, 0.5 mM dithiothreitol [DTT], 20 mM β-glycerophosphate, 0.1 mM Na_3VO_4, 2 μg of leupeptin per ml, 0.5 mM phenylmethylsulfonyl fluoride). The lysate was rotated at 4°C for 30 min and cleared by centrifugation, and the protein concentration was determined by the Bio-Rad protein assay. Extracts were then diluted so that the buffer contained 20 mM HEPES (pH 7.8), 75 mM NaCl, 2.5 mM $MgCl_2$, 0.1 mM EDTA, 0.05% Triton X-100, 0.5 mM DTT, 20 mM β-glycerolphosphate, 0.1 mM Na_3VO_4, 1 μg of leupeptin per ml, and 0.5 mM phenylmethylsulfonyl fluoride. Approximately 1 mg of extract was incubated in a volume of about 10 ml with 5 μg of GST-FosB fusion protein for 2 to 3 h at 4°C. The samples were washed four times with wash buffer (20 mM HEPES [pH 7.8], 50 mM NaCl, 2.5 mM $MgCl_2$, 1 mM DTT, 0.1 mM EDTA, 0.05% Triton X-100, 20 mM β-glycerolphosphate, 0.1 mM Na_3VO_4). The beads were washed once with kinase buffer (20 mM HEPES [pH 7.6], 20 mM $MgCl_2$, 2 mM DTT, 20 mM β-glycerolphosphate, 0.1 mM Na_3VO_4) and resuspended in 30 μl of kinase buffer containing 10 μCi of [γ-^{32}P]ATP. After 20 min at 30°C, the reaction was terminated by several washes in wash buffer. The proteins were then separated by SDS-PAGE, and bands were eluted from the dried gel. Peptide mapping was carried out as described previously (6), with minor changes. Because there are no predicted trypsin cleavage sites in the FosB C terminus, we used chymotrypsin for mapping experiments. Resolution of the FosB-derived peptides was poor when electrophoresis was carried out at pH 1.9; therefore, electrophoresis was carried out in pH 8.9 buffer at 1,000 V for 25 min. Chromatography was carried out in phosphochromatography buffer (6).

In some experiments, GST-FosB fusions harboring the mutations V295M and V305M were labelled as described above. The proteins were blotted to nitrocellulose and digested with cyanogen bromide as described previously (6). The resulting peptides were then separated by electrophoresis at pH 1.9 and chromatography as described above.

Focus formation assays. Focus formation assays were carried out as previously described (38). Briefly, Ψ-2 cells were transfected with SLX-CMV-FosB plasmid DNA (or the appropriate derivatives). Supernatants were collected 2 days later and used to infect 208F cells. Twenty-four hours after infection, cells were split and monitored for the appearance of G418-resistant ($G418^r$) colonies and transformed foci of cells. The assays were carried out three times in duplicate; values represent the averages of the three experiments.

RESULTS

Proline 298 is critical for transcriptional activation by the FosB C terminus. Transcriptional activation and transformation by the FosB protein require the functional integrity of the C-terminal activation domain (Fig. 1 shows a schematic diagram). As a first step in defining the molecular basis of function of this domain, we have undertaken a detailed mutational analysis. The C-terminal region of FosB (amino acids 226 to 338) functions as a potent activation domain when fused to the DNA binding domain of the yeast protein Gal4. We initially analyzed transcriptional activation by a series of Gal4-FosB fusion proteins harboring deletions within the FosB sequences. These fusion proteins were analyzed for activity by expression

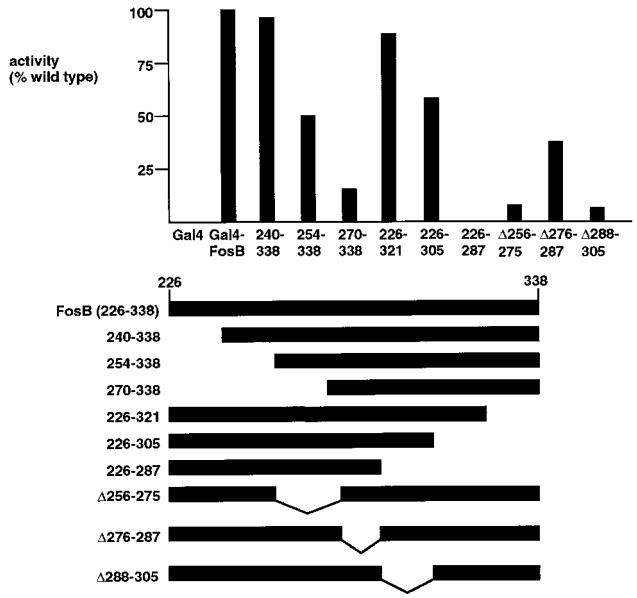

FIG. 2. Deletion analysis of the FosB C-terminal activation domain. A Gal4-FosB (amino acids 226 to 338) fusion protein or deletion derivatives were analyzed for the ability to activate the Gal4 reporter construct pG5B-CAT, which harbors five Gal4 binding sites, by transient expression in 208F cells. Forty hours after transfection, CAT activity was determined as described in Materials and Methods. All values are normalized to those for a cotransfected *lacZ* expression plasmid and represent the averages of four separate determinations, with Gal4-FosB assigned a value of 100%. Schematic diagrams of the deletion mutations are shown.

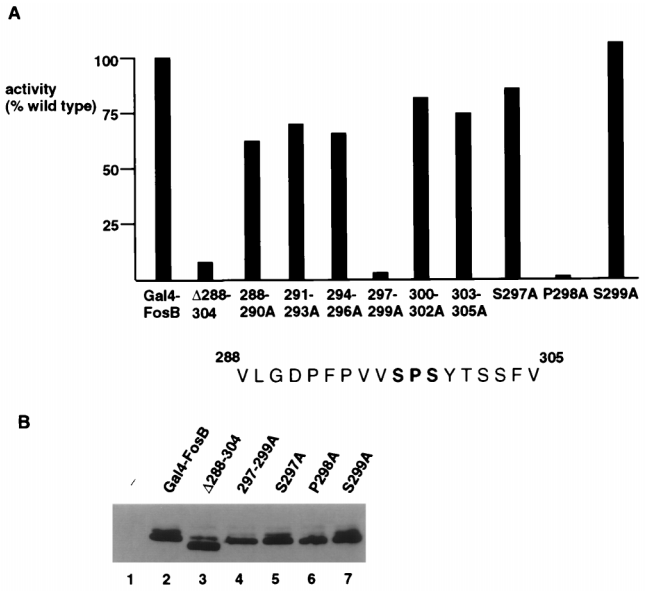

FIG. 3. Alanine substitution analysis of the FosB C-terminal activation domain. (A) Alanine substitutions were generated across the region spanning amino acids 288 to 305 of the FosB protein in the context of a Gal4-FosB fusion protein. The alanine substitution mutants were analyzed for the ability to activate a Gal4 reporter construct by transient expression in 208F cells as described in the legend to Fig. 2. The amino acid sequence in the region from 288 to 305 is shown in single-letter code, with the residues sensitive to mutation shown in boldface. (B) Expression of Gal4-FosB proteins in transiently transfected 293 cells. Plasmids encoding the appropriate fusion proteins were expressed in 293 cells; 40 h later, the expression was analyzed by immunoblotting with an anti-Gal4 antibody (Santa Cruz).

in 208F fibroblasts, a cell line sensitive to transformation by FosB protein, so that transcriptional activation could be correlated with transformation. When these mutant Gal4-FosB proteins were characterized for the ability to activate a reporter construct containing linked Gal4 binding sites, we identified two discrete regions of the protein that were critical for transcriptional activation (Fig. 2). Amino acids 256 to 275 contain a proline-rich sequence that we and others have previously identified as being critical for transcriptional activation by the FosB C terminus (26, 37). In addition, amino acids 288 to 305 are crucial for transcriptional activation, as deletion of these sequences results in a fusion protein (Δ288-305) that retains only 8% of wild-type activity. All other regions of the C terminus can be deleted as blocks of 20 amino acids and still form Gal4-FosB fusion proteins that retain significant activity. The inability of Gal4-FosB Δ288-305 to activate transcription is not due instability of the mutant protein, as it is expressed at levels comparable to that of the wild-type protein (Fig. 3B).

To begin to define how amino acids 288 to 305 might contribute to transcriptional activation by this fusion protein, we generated a series of alanine scanning mutations in this region. Residues in this region were changed to alanine in sets of three. The analysis of this set of mutants showed that a single mutant, 297-299A, was defective in transcriptional activation, while all other mutant proteins activated transcription at near wild-type levels (Fig. 3A). We then mutated residues 297 to 299 to alanine individually; analysis of these mutant fusion proteins showed that a single substitution, P298A, resulted in a marked decrease in transcriptional activation, while substitution of serines 297 and 299 individually to alanine did not markedly impair activity (Fig. 3A). Thus, the lack of activity of the mutant 297-299A could be accounted for by the mutation of proline 298. When the expression of the different fusion proteins was analyzed by Western blotting, we noted that the Gal4-FosB gave rise to two bands; the slower-migrating species accounts for 5 to 10% of the total protein and presumably arises by phosphorylation (Fig. 3B). The abundance of this slower-migrating species was markedly reduced, but not completely eliminated, when substitutions of P298 were present (Fig. 3b, lanes 4 and 6).

Proline 298 is critical for the phosphorylation of neighboring residues. The primary sequence in this region and the analysis of the expression of the fusion proteins suggested a role for proline 298; this proline might direct the phosphorylation of the surrounding serines by a proline-directed kinase. The presence of hydrophobic residues preceding the serine at position 297 is consistent with the known specificities of this family of enzymes (1, 12). Additionally, our data suggest that if P298 directs the activity of a kinase that phosphorylates and activates FosB, then neither S297 nor S299 serves as the sole phosphorylation site required for activation.

To determine which, if any, serines or threonines in this region are phosphorylated, we turned to phosphopeptide mapping. 293 cells transiently transfected with FosB expression plasmids were metabolically labelled with [^{32}P]orthophosphate. The FosB protein was then immunoprecipitated from cell extracts and purified by SDS-PAGE (Fig. 4A). FosB protein was eluted from the gel, digested with chymotrypsin (there are no consensus trypsin cleavage sites in this region), and resolved by two-dimensional phosphopeptide mapping. Because the different peptides were not well resolved by electrophoresis at pH 1.9, all experiments shown used electrophoresis at pH 8.9. Two-dimensional phosphopeptide mapping of the wild-type FosB protein labelled in vivo shows the presence of several peptides (Fig. 4B, peptides a to d), along with a large

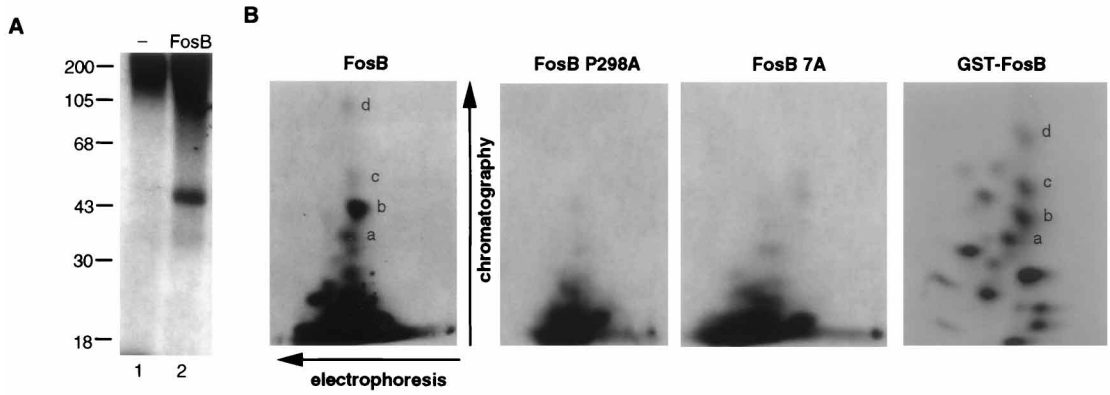

FIG. 4. Phosphopeptide mapping of FosB proteins labelled in vivo. (A) Human 293 cells were transiently transfected with plasmid SLX-CMV FosB; 40 h after transfection, cells were labelled with [^{32}P]orthosphosphate, and FosB proteins were isolated by immunoprecipitation. The immunoprecipitates were then separated on SDS-PAGE and visualized by autoradiography. Lane 1, untransfected 293 cells; lane 2, 293 cells transfected with SLX-CMV-FosB. Sizes are indicated in kilodaltons. (B) Wild-type or mutant FosB proteins were expressed in 293 cells and metabolically labelled with [^{32}P]orthophosphate. The proteins were purified by immunoprecipitation and SDS-PAGE as described above. Phosphopeptide mapping of the proteins was then carried out as described in Materials and Methods. The right panel is a map of GST-FosB(270-338) labelled in vitro; the other panels are maps of wild-type or the indicated mutant FosB proteins labelled in vivo. Peptides a to d are indicated.

amount of material that is poorly resolved under these conditions. Maps of FosB P298A show a complete loss of peptides a to d, suggesting that phosphorylation of these sites is dependent on the presence of a proline residue at position 298. Because mutation of proline 298 was also associated with diminished transcriptional activation by Gal4-FosB, we focused our efforts on identification of the residues that are phosphorylated in peptides a to d.

Several complications in the mapping process were expedited by the ability to map recombinant proteins in vitro. The in vitro substrate was a fusion protein in which amino acids 270 to 338 of FosB were fused to GST (GST-FosB). Using a modified version of the protocol of Hibi and coworkers (14), we incubated the different fusion proteins with a cytoplasmic extract and then labelled them by the associated kinases in the presence of [γ-^{32}P]ATP. When the labelled material was separated by SDS-PAGE and visualized by autoradiography, we found that GST-FosB was labelled in a manner that was dependent on prior incubation with the cell extract (Fig. 5A). The phosphorylated protein was retarded in mobility on SDS-PAGE to a similar degree as the Gal4-FosB protein (data not shown), suggesting that these phosphorylations can give rise to the observed mobility shift. The labelled fusion proteins were then analyzed by two-dimensional peptide mapping. Our initial experiments showed that peptides a to d are present in maps of GST-FosB, consistent with the idea that these peptides are derived from this region of the FosB molecule (Fig. 4B and 5B). Furthermore, these peptides are not present in the map of GST-FosB Δ288-305 or GST-FosB P298A (Fig. 5B). These results show that peptides a to d are phosphorylated in vitro in a manner that is dependent on the presence of P298.

To identify the residues that are phosphorylated in peptides a to d, we used the same assay to analyze a series of GST-FosB mutant proteins. It is important to note that this in vitro assay functions as a two-step assay, in which phosphorylation is contingent on prior association of a kinase. The absence of individual peptides in this assay can result either from failure of the kinase to associate with the substrate or from mutation of the phosphoacceptor residues to nonphosphorylatable amino acids. However, the generation of phosphopeptides with mobility different from that of mutant substrates demonstrates that (i) the relevant kinase still associates with the substrate and (ii) the mutated residues are contained within the phosphopeptide. Mutation of S297 and S299 to alanine (S297/299A) resulted in increased migration in the chromatographic dimension of peptides a to d. This result demonstrates that peptides a to d all contain S297 and S299, and the change in mobility is consistent with the introduction of less hydrophilic alanine residues. Likewise, mutation of T301, S302, and S303 to alanine resulted in a similar shift in the migration of peptides a to d, demonstrating that these residues are also contained in peptides a to d. In each case, all of the peptides are present, demonstrating that none of the mutated residues is absolutely required as a phosphoacceptor site in the generation of these peptides. Mutation of all five of these residues simultaneously to alanine (5A) results in a complete loss of peptides c and d and a further increase in the mobility of peptides a and b in the chromatographic dimension. This result demonstrates that phosphorylation of the clustered serine and threonine residues between amino acids 297 and 303 gives rise to peptides c and d and further shows that peptides a and b contain these residues. Mutation of T282 and S284 to alanine alters the mobility of peptides a and b, while peptides c and d are unaffected, demonstrating that peptides a and b contain T282 and S284. Mutation of T282 and S284 to alanine in addition to S297, S299, T301, S302, and S303 (7A) results in the complete loss of peptides a to d, demonstrating that peptides a and b contain these residues as well as the cluster of residues that includes S297, S299, T301, S302, and S303. Taken together, the results show that peptides a to d are derived from a series of partial proteolytic cleavages of the region surrounding P298. They further show that no specific phosphoacceptor residue is required for the generation of any particular phosphopeptide. Instead, the kinase(s) that phosphorylates this region in vitro appears to be able to phosphorylate nearby phosphoacceptor sites in the event of mutation of any individual site. Phosphoamino acid analysis of peptides a through d demonstrated that in each case only phosphoserine was detectable (data not shown), excluding T282 and T301 as sites of phosphorylation. In addition, maps of GST-FosB proteins harboring alanine substitutions of all other serine or threonine residues in the FosB portion of the fusion protein contain peptides a to d, and peptide maps of the FosB portion of the fusion protein cleaved with thrombin revealed the presence of peptides a to d (data

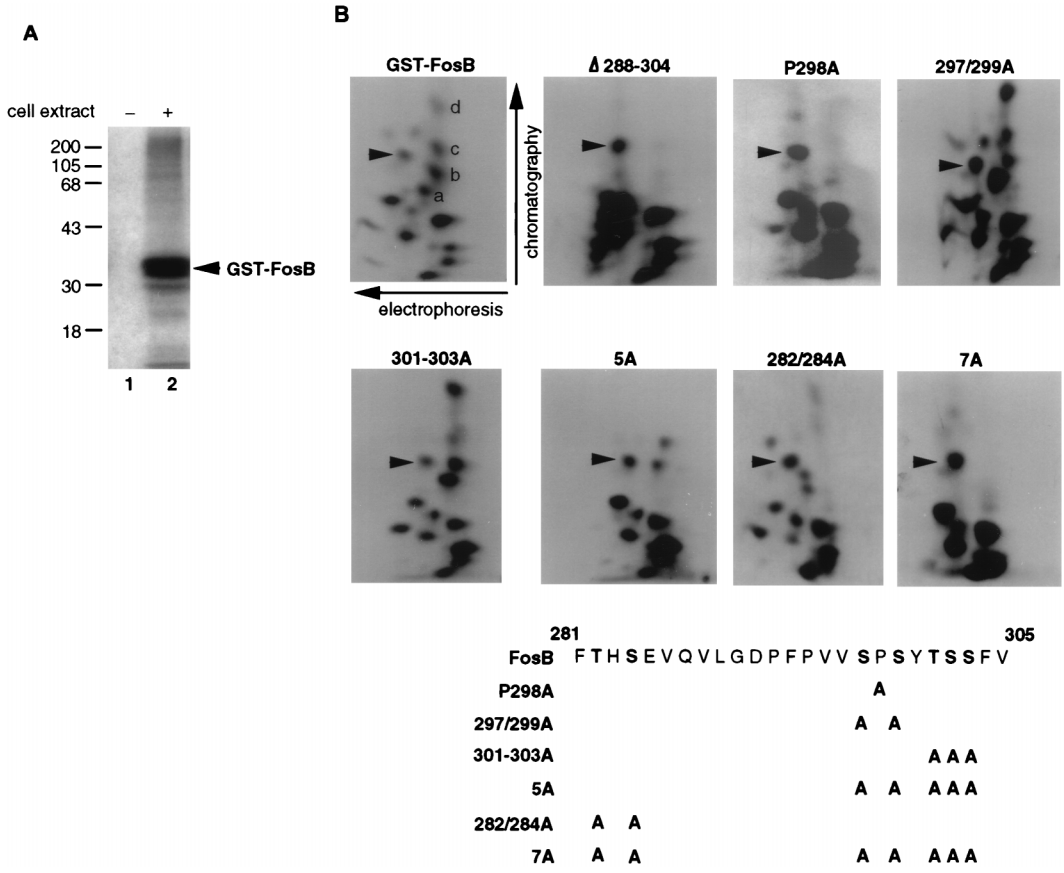

FIG. 5. Phosphopeptide mapping of FosB C-terminal sites labelled in vitro. (A) GST-FosB (amino acids 270 to 338) protein immobilized on glutathione-Sepharose beads was incubated with whole-cell extract from 208F cells and washed. The beads were then incubated in kinase buffer and [γ-^{32}P]ATP; after 20 min, the kinase reaction was stopped, and the beads were washed (see Materials and Methods). The proteins were separated on SDS-PAGE and visualized by autoradiography. Lane 1, no cell extract; lane 2, plus cell extract. The positions of the molecular mass standards are indicated. Sizes are indicated in kilodaltons. (B) GST-FosB proteins labelled as described above were eluted from the gel, digested with chymotrypsin, and subjected to two-dimensional phosphopeptide mapping. The arrow in each of the panels indicates a peptide that is common to all of the maps and is generated by phosphorylation of the extreme C-terminal peptide that includes serines 332 and 334. The peptides labelled a to d are derived from the region containing amino acids 281 to 305. The sequence in this region is shown in single-letter amino acid code, and the sites of alanine substitution are shown.

not shown). The results are consistent with the hypothesis that the serine residues identified (S284, S297, S299, S302, and S303) are phosphorylated in vitro to give rise to peptides a to d; mutation of a subset of these phosphoacceptor sites to alanine results in the phosphorylation of alternate sites. The relationship of these peptides to each other suggests that they do not derive from the processive phosphorylation of serine residues within this cluster but instead are the result of a series of partial proteolytic digestions in which each peptides contains a single phosphoserine residue. These results do not permit any conclusions regarding either the biochemical nature of the requirement for the proline residue at position 298 or regarding the relationship of the kinase(s) that mediates phosphorylation of these residues in vitro with those that phosphorylate FosB protein in vivo.

To verify that serine residues clustered in the region of P298 are sites of phosphorylation of FosB protein in vivo, we performed two-dimensional peptide mapping experiments on a FosB mutant protein (FosB 7A) labelled in vivo. Mutation of the cluster of candidate phosphoacceptor sites resulted in the disappearance of peptides a to d (Fig. 4B). This result confirms that the cluster of serine residues identified by the in vitro experiments (S284, S297, S299, S302, and S303) are sites of phosphorylation in vivo. Furthermore, combined with the earlier analysis, the results show that phosphorylation of these sites is dependent on the presence of proline 298, a result that correlates with the observation that transcriptional activation by Gal4-FosB is inactivated by mutation of this residue. It is important to note that we have not determined whether phosphorylation in this region is confined to a single serine residue in the wild-type protein and, if this is so, which serine is phosphorylated.

Transcriptional activation and transformation by FosB require C-terminal phosphorylation. Previous analysis showed that mutation of proline 298 eliminated the efficiency of phosphorylation of nearby residues in vivo and reduced transcriptional activation by the FosB C-terminal domain. If Gal4-FosB P298A is impaired in transcriptional activation solely as a consequence of impaired phosphorylation, then mutation of the phosphoacceptor sites to nonphosphorylatable residues should also impair transcriptional activation. To test this hypothesis, we analyzed transcriptional activation by Gal4-FosB fusion proteins harboring mutations in all or a subset of the phosphoacceptor residues identified. This analysis shows that simultaneous alanine substitutions in all of the phosphorylatable serine residues results in a Gal4-FosB protein that is severely compromised for transcriptional activation, but no single serine residue is absolutely required (Fig. 6A). The results

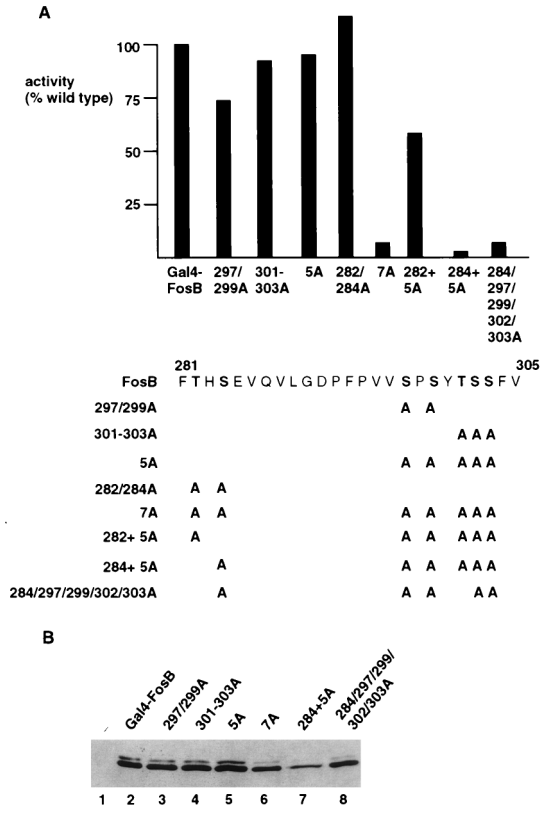

FIG. 6. Analysis of the effects of phosphorylation site mutations in the FosB C-terminal activation domain. (A) Different phosphoacceptor residues were mutated to alanine in the context of a Gal4-FosB fusion protein. The mutants were analyzed by transient transfection for the ability to activate a Gal4 reporter construct in 208F cells as described in the legend to Fig 3. The sequence of amino acids 281 to 305 is shown in single-letter amino acid code, and the positions of the alanine substitutions in the different proteins are shown. (B) Expression of Gal4-FosB proteins in transiently transfected 293 cells. Plasmids encoding the different fusion proteins were expressed in 293 cells; 40 h later, the expression was analyzed by immunoblotting with an anti-Gal4 antibody.

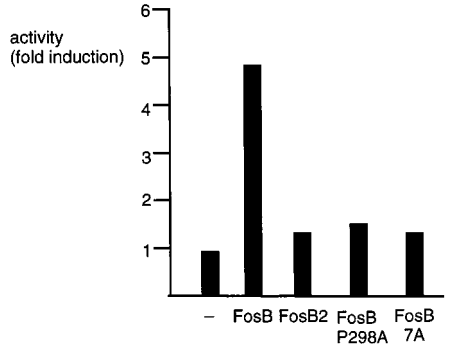

FIG. 7. Analysis of phosphorylation site mutations in the FosB protein. Plasmids encoding the indicated FosB proteins were cotransfected into F9 cells along with plasmids encoding c-Jun protein, an AP-1-based reporter construct (pT3B-CAT; see Materials and Methods), and a *lacZ* expression plasmid as a control; 40 h after transfection, CAT activity was determined.

show that it is not the number of alanine substitutions but the specific sites that are important, as a mutant with alanine substitutions of S284, S297, S299, S302, and S303 is inactive, while another mutant with five alanine substitutions (Fig. 5A; substitutions of S297, S299, T301, S302, and S303) retains some, although not the wild-type, level of activity. The results are consistent with the phosphopeptide mapping data and suggest that transcriptional activation by the FosB C-terminal domain requires the phosphorylation of any of several nearby serine residues, while mutation of any individual residue is compatible with at least partial activity. Western blotting with an anti-Gal4 antibody confirmed that all of the Gal4-FosB fusion proteins were expressed to equivalent degrees. Furthermore, mutants harboring alanine substitutions of the relevant serine residues showed decreased accumulation of the slower-migrating species of the protein, suggesting, but not proving, that the slower-migrating form arises from phosphorylation (Fig. 6B; compare lane 2 with lanes 6 to 8). These experiments demonstrate that phosphorylation of a subset of serine residues is required for efficient transcriptional activation by the FosB C-terminal activation domain but that no individual serine is critical for this function.

Previous work has shown that transcriptional activation and focus-forming activity by the native FosB protein are dependent on the activity of the C-terminal activation domain (37). To determine if these properties are dependent on the presence of C-terminal phosphorylation sites, we introduced alanine substitution mutations into either proline 298 or the surrounding serine phosphoacceptor residues. These FosB mutant proteins were analyzed for the ability to activate expression, along with c-Jun, of a cotransfected reporter construct in F9 cells, which contain relatively low levels of endogenous AP-1 activity. The results show that transcriptional activation by the nonphosphorylatable derivatives is impaired to a level similar to that observed for FosB2, which does not contain the C-terminal sequences, and demonstrate a role for the C-terminal phosphorylation sites in transcriptional activation by FosB (Fig. 7). The FosB mutant proteins were analyzed for transforming activity by performing focus formation assays in 208F fibroblasts. This analysis shows that mutation of either P298 or all of the surrounding phosphorylation sites results in mutant FosB proteins that are severely impaired in focus-forming activity (Table 1). Consistent with the analysis of transcriptional activation, mutation of some, but not all, serines in this region resulted in FosB proteins that retained significant transforming activity. Together, the results show that the biologic functions of FosB, transcriptional activation and neoplastic transformation, require phosphorylation of the serine residues identified above.

TABLE 1. Transformation by FosB proteins

Virus	No. of foci	No. of G418^r colonies	Focus-forming activity[a]
None	0	0	0
FosB	216	348	100
FosB2	0	411	0
FosB P298A	14	293	8
FosB S297/299A	57	244	38
FosB 301-303A	176	220	129
FosB 5A	12	188	10
FosB 282/284A	141	192	118
FosB 7A	12	185	11

[a] 208F cells were infected with recombinant retroviruses directing expression of the indicated FosB proteins. Twenty-four hours after infection, the cells were split into four plates; two were monitored for the appearance of foci, and two were selected with G418. G418^r colonies and foci of transformed cells were counted 12 to 14 days after infection. The focus-forming activity is the number of foci divided by the number of G418^r colonies; the data are normalized such that wild-type FosB has a value of 100. The results were repeated in duplicate three times; the results are the averages of the three experiments.

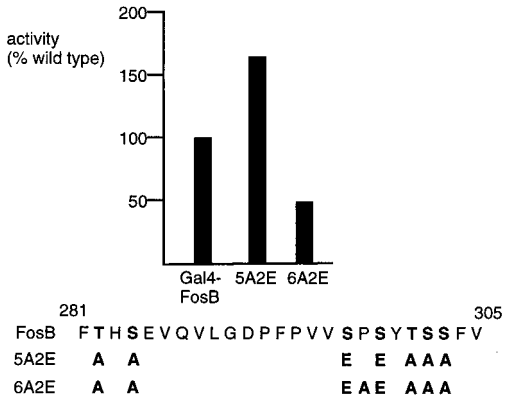

FIG. 8. Effects of glutamic acid substitution mutations on transcriptional activation by Gal4-FosB. Mutant Gal4-FosB proteins containing the indicated glutamic acid and alanine substitutions were analyzed for the ability to activate a Gal4 reporter construct as previously described. Values are expressed relative to those for the wild-type Gal4-FosB fusion protein. The sequence of the mutant proteins is shown in single-letter amino acid code.

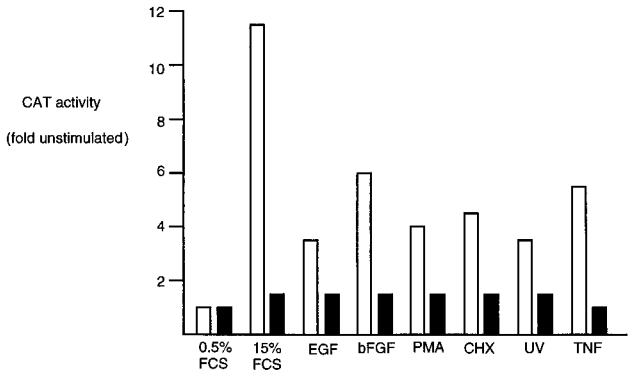

FIG. 9. Transcriptional activation by Gal4-FosB is not enhanced by activators of ERK or SAP kinases. Plasmids encoding Gal4-FosB or Gal4-Elk1, the Gal4 reporter construct pG5B-CAT, and a control *lacZ* expression plasmid were introduced into 208F cells. Following transfection, the cells were place in medium containing 0.5% FCS. After an additional 36 h, the cells were treated with 15% FCS, epidermal growth factor (EGF; 20 ng/ml), basic fibroblast growth factor (bFGF; 60 ng/ml), phorbol myristate acetate (PMA; 100 ng/ml), cycloheximide (CHX; 10 μg/ml for 2 h), UV light (40 J/m^2, 254 nm), or TNF-α (50 ng/ml); CAT activity was analyzed 8 h after treatment. All values are relative to cells maintained in 0.5% FCS, and values were normalized to β-galactosidase activity. White bars represent activation by Gal4-Elk1, and black bars represent activation by Gal4-FosB.

If phosphorylation of serines 284, 297, 299, 302, and 303 is critical for function of the FosB C-terminal activation domain, then mutation of some of these residues to negatively charged residues (glutamic acid) might result in a constitutively active C-terminal activation domain. To test this idea, we generated two different mutants. The first contains alanine substitution of all of the candidate phosphoacceptor sites except for glutamic acid substitutions at amino acids 297 and 299 (Gal4-FosB 5A2E). The second contains all of the substitutions described above along with an alanine substitution of P298 (Gal4-FosB 6A2E). The number of glutamic acid substitutions was limited to two for two reasons: first, our peptide mapping data do not suggest the addition of multiple phosphates within the same molecule; and second, the substitution of glutamic acid residues at multiple sites might result in spurious activation simply by the creation of a highly acidic region. Mutant 5A2E contains activity greater than that of the wild-type protein, while 6A2E has about 65% of wild-type activity (Fig. 8). The results are consistent with the hypothesis that the introduction of negative charge into the C terminus of FosB proteins, either by phosphorylation or by mutation, is critical for transcriptional activation. Furthermore, the results suggest that the only function of P298 is to mediate the phosphorylation of the surrounding serine residues, as mutation of this residue is tolerated in the presence of glutamic acid substitutions.

The serine residues surrounding proline 298 resemble consensus sites for the MAP kinase family of kinases. As both ERK kinases and SAP kinases are known to regulate AP-1 activity, we considered the possibility that these kinases phosphorylate the FosB C-terminal domain. Two experiments argue against this possibility, however. First, reporter gene activation by Gal4-FosB is not enhanced by any of several activators of either the ERK or SAP kinases, including serum, growth factors, phorbol esters, the protein synthesis inhibitor cycloheximide, UV light (40 J/m^2), and TNF-α (Fig. 9). In contrast, transcriptional activation by Gal4-Elk1, which is subject to phosphorylation and activation by these stimuli, is observed. Second, the MAP and SAP kinases do not bind to GST-FosB under the conditions used in the in vitro kinase assay (data not shown). These experiments suggest that the ERK and SAP kinases do not regulate activity of the FosB C-terminal domain by phosphorylation. Therefore, activation of the FosB C-terminal activation domain is presumably due to phosphorylation by a different kinase(s).

DISCUSSION

Transcriptional activation by Fos proteins is dependent on regions outside the bZIP motif, including a C-terminal activation domain that is a major determinant of their biologic activities (16, 26, 28, 37–39). Using a detailed mutational analysis, we have shown that transcriptional activation by the FosB C-terminal activation domain is dependent on phosphorylation within a cluster of serine residues, including serines 284, 297, 299, 302, and 303. While we have not determined whether phosphorylation of the wild-type protein occurs at a single serine residue and, if it does, which serine is phosphorylated, our data suggest that no individual serine residue is required for either phosphorylation of or transcriptional activation by the FosB C terminus. Instead, these sites appear to be functionally redundant, in that phosphorylation of any of them appear to be compatible with transcriptional activation. Mutations in other residues that impair phosphorylation (i.e., P298) also diminish transcriptional activation by the FosB C-terminal domain. The results demonstrate that FosB is synthesized as an inactive protein that must be phosphorylated for full biologic activity and suggest a potential mechanism for the integration of multiple growth regulatory signals by a single transcription factor.

Regulation of the FosB C terminus. Our data demonstrate that the FosB C terminus is phosphorylated in a manner that is dependent on the integrity of P298; this phosphorylation is required for the FosB C terminus to mediate transcriptional activation. No individual serine is required for this region to become phosphorylated and activated, suggesting that the kinase(s) that phosphorylates this region can utilize any of the identified serines as phosphoacceptor sites. Such an arrangement of phosphorylation sites, wherein mutation of one phosphorylation site results in the phosphorylation of a nearby site, has previously been observed (35). In the case of FosB, it is unusual that a single proline appears to direct the phosphorylation of several different sites, one of which (S284) is sepa-

rated from P298 by 14 amino acids in the primary sequence. Definition of the biochemical basis of the requirement for a proline residue at position 298 awaits identification of the kinase(s) that carries out this phosphorylation.

We do not know the stoichiometry of phosphorylation of the FosB C terminus in vivo. Immunoblotting of Gal4-FosB fusion proteins shows that a slower-migrating form of the protein is observed, and the abundance of this species is markedly reduced with nonphosphorylatable mutants, suggesting that much of this species derives from phosphorylation of the C-terminal serine residues. As this slower-migrating form is a minor species under all conditions tested, we believe that in growing cells, only 5 to 10% of the protein is phosphorylated at these C-terminal sites. Activation of the appropriate kinase, by a currently unknown stimulus, presumably would induce a higher level of phosphorylation.

No individual serine residue in the FosB C terminus is absolutely required for transcriptional activation; instead, our mapping experiments indicate that the kinase responsible for the activating phosphorylation can modify any of several potential phosphoacceptor sites. In this regard, the FosB C-terminal kinase(s) is somewhat promiscuous. This result is analogous to phosphorylation of the G_1 cyclin CLN2 of *Saccharomyces cerevisiae* by the proline-directed kinase CDC28. Rapid turnover of CLN2 is dependent on phosphorylation by CDC28, but no individual serine or threonine phosphorylation sited is required; instead, many appear to contribute to the rapid proteolysis (22). Alternatively, several different kinases may phosphorylate different subsets of serine residues within this region. The mechanism by which the activity of the FosB C-terminal domain is increased by phosphorylation is not clear, but analysis of the glutamic acid substitution mutants suggests that negative charge alone is sufficient to mediate this activation.

The proline-directed family of kinases includes the ERK and SAP kinases, two families of kinases that have already been shown to regulate AP-1 activity (15, 25, 29, 32, 33). However, our data show that transcriptional activation by the FosB C-terminal domain is not activated by activators of either the ERK or SAP kinases. Furthermore, these kinases do not associate with GST-FosB under conditions in which the in vitro labelling is carried out. Taken together, the results argue strongly that the FosB C terminus is phosphorylated by an enzyme that is distinct from the ERK and SAP kinases. However, phosphorylation is dependent on the presence of a proline-based motif in the substrate.

The identity of the FosB C-terminal kinase is unknown, but the associated kinase used to label the protein in vitro shares two important properties with the enzyme that phosphorylates the C terminus in vivo. First, both enzymes phosphorylate the same subset of C-terminal residues, and second, the ability of both enzymes to phosphorylate the FosB C terminus is dependent on the presence of P298. This finding suggests, but does not prove, that the enzyme that associates with the FosB C terminus in vitro is either related or identical to the in vivo FosB kinase. This result may prove useful in identifying the enzyme responsible for activation of FosB in vivo.

Mechanism of AP-1 regulation. The mechanism of AP-1 activation is complex; one level of regulation involves an increase in the abundance of different AP-1 components in response to growth factors. In the best-defined case, activation of the c-Fos promoter is mediated by phosphorylation of the transcription factor Elk-1 by the kinases ERK-1 and ERK-2 (10, 15, 25). Although the FosB promoter is not characterized as well as the c-Fos promoter, it contains a serum response element, and the FosB gene is induced by canonical inducers of serum response element activity, such as serum, growth factors,

```
FosB   D P F     P   V V     S P S       Y T S S F V L
c-Fos  E P L C T P   V V T C T P G C T T Y T S S F V F
Fra-1  E A L H T P T L M   T T P S L T P F T P S L V F
Fra-2  E P L H T P I V V T S T P A V T P G T S N L V F
```

FIG. 10. Sequence alignment of Fos proteins in the region surrounding the FosB C-terminal phosphorylation sites. The equivalent of FosB P298 is shown in boldface. The residues shown in single-letter amino acid code are mouse FosB 291-306, mouse c-Fos 321-343, human Fra-1 201-223, and human Fra-2 259-282. The proline residue corresponding to FosB P298 is shown in boldface; other (S/T)P sites, which are potential sites of phosphorylation, are underlined.

and phorbol esters (23, 40). Thus, one level of activation of AP-1 activity is due to the increase in the synthesis of Fos proteins, as well as Jun proteins, that occurs in the presence of growth factors, and this increase in the level of individual AP-1 proteins is translated into an increase in the AP-1 DNA binding activity.

In addition to an increase in the amount of AP-1 proteins, AP-1 activity is regulated by posttranslational modification. The best-defined example of regulation of AP-1 activity at the posttranslational level is the increase in c-Jun activity induced by phosphorylation within the transactivation domain that is mediated by phosphorylation of serines 63 and 73 by the SAP kinases (also known as JNKs) (9, 21, 29, 32). Although all of the Fos family of proteins are highly phosphorylated, the only well-described form of regulation of Fos proteins by phosphorylation is the phosphorylation of c-Fos at serine 232 in response to *ras* activation (8). We have shown here that activity of the FosB protein is dependent on phosphorylation of a cluster of serine residues in the C-terminal activation domain. The essential features of this region, a proline surrounded by potential phosphoacceptor residues, is conserved in all of the Fos family proteins, suggesting that this may be a common mode of regulation of Fos proteins (Fig. 10). Many of the other family members contain additional (S/T)P motifs in the immediate vicinity.

The biologic regulation that underlies this phosphorylation is not clear. Our data suggest that the stoichiometry of phosphorylation is relatively low in growing 208F cells. The ability to increase phosphorylation of Fos proteins in response to a particular stimulus would be predicted to increase signaling by AP-1 proteins. Thus, phosphorylation of the FosB C terminus may serve as a molecular mechanism for integration of signals at the level of a single transcription factor. It will be interesting to elucidate the signaling pathways that control phosphorylation of the FosB C terminus.

We have characterized a role for this regulation in the function of FosB in fibroblasts, where it regulates growth control. In the intact animal, FosB has been shown to be critical for certain behaviors, including maternal nurturing (7). It remains to be determined if phosphorylation of FosB functions as a mechanism to regulate signaling in the nervous system, where the ability to associate different biochemical events is likely to be important.

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The first two authors contributed equally to this study.

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