Phosphorylation of the TAL1 Oncoprotein by the Extracellular-Signal-Regulated Protein Kinase ERK1

JUIN-TSUEY CHENG,¹ MELANIE H. COBB,² AND RICHARD BAER^{1*}

Departments of Microbiology¹ and Pharmacology,² University of Texas Southwestern Medical Center,
5323 Harry Hines Boulevard, Dallas, Texas 75235

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Alteration of the *TAL1* gene is the most common genetic lesion found in T-cell acute lymphoblastic leukemia. *TAL1* encodes phosphoproteins, pp42^{TAL1} and pp22^{TAL1}, that represent phosphorylated versions of the full-length (residues 1 to 331) and truncated (residues 176 to 331) *TAL1* gene products, respectively. Both proteins contain the basic helix-loop-helix motif, a DNA-binding and protein dimerization motif common to several known transcriptional regulatory factors. We now report that serine residue 122 (S122) is a major phosphorylation site of pp42^{TAL1} in leukemic cell lines and transfected COS1 cells. In vivo phosphorylation of S122 is induced by epidermal growth factor with a rapid time course that parallels activation of the ERK/MAP2 protein kinases. Moreover, S122 is readily phosphorylated in vitro by the extracellular signal-regulated protein kinase ERK1. These data suggest that TAL1 residue S122 serves as an in vivo substrate for ERK/MAP2 kinases such as ERK1. Therefore, S122 phosphorylation may provide a mechanism whereby the properties of TAL1 polypeptides can be modulated by extracellular stimuli.

Alteration of the *TAL1* proto-oncogene is the most common genetic lesion known to be associated with T-cell acute lymphoblastic leukemia (T-ALL). Almost 25% of T-ALL patients harbor tumor-specific rearrangements of the *TAL1* gene that are the result of either chromosome translocation or local DNA recombination (4-7, 16-18, 20, 21, 37, 45). Rearranged *TAL1* alleles are transcriptionally active in T-ALL cells, and thus inappropriate expression may be the mechanism by which *TAL1* contributes to leukemogenesis.

The *TAL1* gene encodes two phosphoproteins, the full-length gene product, pp42^{TAL1} (amino acid residues 1 to 331), and a truncated polypeptide, pp22^{TAL1} (residues 176 to 331) (18). Both proteins contain the basic helix-loop-helix (bHLH) motif, a DNA-binding and protein dimerization domain common to several known transcriptional regulatory factors (33, 34). The bHLH domain of TAL1 forms heterodimers in vitro with E47 and E12, two bHLH proteins encoded by the E2A gene (27). Since the resultant heterodimers (TAL1/E47 and TAL1/E12) specifically recognize the E-box sequence (CANNTG) of eucaryotic transcription enhancers, TAL1 may function in vivo as a transcriptional regulatory factor (27). The bHLH domain of TAL1 is especially related to those encoded by *LYL1* and *TAL2*, distinct genes that were also identified on the basis of chromosomal rearrangement in human T-cell leukemia (32, 44, 46). Hence, TAL1, TAL2, and *LYL1* constitute a discrete subgroup of bHLH proteins, each of whose members can potentially promote the development of T-ALL (46).

Protein phosphorylation can profoundly affect the functional properties of known transcriptional regulatory factors, including their DNA-binding activity, subcellular localization, and transactivation potential (10, 28, 29). Since the enzymatic activities of the responsible protein kinases are often stimulated by extracellular signals, phosphorylation of transcription factors is a potent mechanism by which nuclear transcription patterns can be modulated by the extracellular environment. The cellular response to growth factor stimu-

lation includes activation of the ERK/MAP2 protein kinases, a family of serine/threonine kinases that are characterized by their ability to phosphorylate microtubule-associated protein 2 and myelin basic protein in vitro (9, 19, 41, 43). Three members of this family, the extracellular signal-regulated kinases 1, 2, and 3 (ERK1, ERK2, and ERK3), were recently identified by molecular cloning (12, 13, 26, 35). In this report, we provide evidence that a major phosphorylation site of pp42^{TAL1} in leukemic cell lines is an in vitro substrate, and possibly an in vivo substrate, for the ERK1 protein kinase. Hence, the properties of TAL1 oncoproteins may be influenced by differential phosphorylation in response to extracellular ligands.

MATERIALS AND METHODS

Tryptic mapping of phosphoproteins. Leukemic cell lines and transiently transfected COS1 cells were metabolically labeled with ³²P_i as described previously (18). TAL1 phosphoproteins were then purified from cell lysates by immunoprecipitation with an anti-TAL1 antiserum and electrophoresis on sodium dodecyl sulfate (SDS)-polyacrylamide gels (18, 23). Purified phosphoproteins were recovered from polyacrylamide gel fragments by electroelution with an Elutrap apparatus (Schleicher & Schuell). The eluted phosphoproteins were oxidized in performic acid, digested with trypsin (Worthington Biochemical Co.), and fractionated in two dimensions essentially as described by Boyle et al. (15). Each tryptic digest was applied to a thin-layer cellulose chromatography plate (Kodak Chromagram sheet 13255), and electrophoresis was performed in the first dimension (1.5 kV for 45 min in pH 1.9 buffer), using an HTLE-7000 apparatus (CBS Scientific). The cellulose plate was dried, and ascending chromatography in isobutyric acid buffer was carried out in the second dimension for 4 h. The plate was then dried in an 80°C incubator prior to autoradiography. Individual phosphopeptides were recovered from cellulose thin-layer plates as described by Boyle et al. (15).

Preparation of ERK1 protein kinase. ERK1 protein kinase was purified from Rat-1 HIRc B cells as described previously

* Corresponding author.

(11). The majority of mapping experiments were performed with material purified through the QAE-Sepharose step; immunoblotting showed that this material contained a predominance of ERK1 protein kinase, with relatively little ERK2. Confirmatory experiments were performed with material purified through the final DEAE-cellulose step and thus contained highly purified ERK1 (specific activity with myelin basic protein as substrate, ~ 0.5 to 1.0 $\mu\text{mol}/\text{min}/\text{mg}$ of protein).

In vitro phosphorylation with ERK1 protein kinase. Bacterial expression vectors encoding glutathione *S*-transferase (GST)-TAL1 fusion proteins were constructed by inserting *TAL1* cDNA sequences into appropriate pGEX plasmid vectors (22, 40). GST-TAL1 fusion proteins were expressed in *Escherichia coli* and purified to homogeneity by affinity chromatography on glutathione-agarose beads as described by Smith and Johnson (40). For each phosphorylation reaction, 3 μg of GST-TAL1 fusion protein was mixed with 3.5 to 5.0 U (1 U = 1 pmol/min) of ERK1 protein kinase. The reaction was started by the addition of 10 μl of an ATP mix giving final concentrations of 30 μM ATP (with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ to give 1.2 cpm/fmol), 15 μM MgCl_2 , 1.5 mM dithiothreitol, and 25 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES; pH 8.0). After 20 min at room temperature, 10 μl of 2 $\times$ sample buffer (0.1 M Tris-HCl [pH 6.8], 2% SDS, 0.004% bromophenol blue, 40% glycerol, 0.3 mM β -mercaptoethanol) were added to stop the reaction. The sample was then boiled for 5 min and analyzed by SDS-polyacrylamide gel electrophoresis.

For in vitro phosphorylation of synthetic peptides, the peptide substrate (TAL1 residues 118 to 134) was resuspended in 50 mM HEPES buffer (pH 8.0). ERK1 protein kinase was added to 15 μl of the resuspended peptide (40 μg), and the phosphorylation reaction was carried out as described above. After 20 min at room temperature, the reaction was stopped by adding 10 μl of concentrated acetic acid. One microliter of the reaction was spotted onto a thin-layer cellulose plate, and the phosphorylated peptide was separated from $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ by ascending chromatography in isobutyric acid buffer.

EGF stimulation of COS1 cells. For epidermal growth factor (EGF) stimulation experiments, COS1 cells were transfected with expression plasmid TAL1/pCMV4 as described previously (18). Forty-eight hours after transfection, the cells were transferred into either serum-free, methionine-free RPMI 1840 medium (ICN Biomedicals) or serum-free, phosphate-free RPMI 1840 medium (GIBCO). After 1 h, the cultures were supplemented with either $[\text{S}^{35}]\text{methionine}$ or $^{32}\text{P}_i$. After 2 h of labeling, the cultures were treated with a saturating concentration (100 ng/ml) of EGF; 10 min later, the cells were lysed in radioimmunoprecipitation assay (RIPA) buffer containing protease inhibitors. EGF was purified from mouse submaxillary glands as previously described (3, 38) and provided as a gift by Rebecca L. Ball.

Phosphatase treatment of immunoprecipitates. Immunoprecipitates were collected on protein A-Sepharose beads as described previously (18). After the last wash with RIPA buffer, the beads were washed twice in bacterial alkaline phosphatase (BAP) buffer (100 mM Tris [pH 8.0], 50 mM MgCl_2 , 0.1% aprotinin) and finally resuspended in 45 μl of fresh BAP buffer. The immunoprecipitates were then treated with 0.7 U of BAP (Sigma) at 30°C for 45 min. The Sepharose beads were washed twice in RIPA buffer and resuspended in 2 $\times$ sample buffer. Samples were boiled for 10 min and fractionated by electrophoresis on an SDS-8 to 15% polyacrylamide gradient gel.

FIG. 1. Tryptic maps of pp42^{TAL1} from human leukemic cell lines. pp42^{TAL1} was purified from $^{32}\text{P}_i$ -labeled cells by immunoprecipitation and SDS-polyacrylamide gel electrophoresis. Tryptic digests of pp42^{TAL1} were applied to thin-layer cellulose plates and fractionated in the first dimension by electrophoresis at pH 1.9 (anode to the left) and in the second dimension by ascending chromatography in isobutyric acid buffer. Tryptic maps of pp42^{TAL1} Jurkat T-ALL cells (A) and K562 erythroleukemia cells (B) are shown. The mobilities of the 2,4-dinitrophenol-lysine (DNP-K) marker in both dimensions are indicated by oval circles.

Eukaryotic expression plasmids and in vitro mutagenesis. Plasmid TAL1m176/pCMV4 encoding truncated TAL1 polypeptides (residues 176 to 331) was constructed by inserting a 0.95-kb *TAL1* cDNA fragment into the *Bgl*III site of the pCMV4 vector (2). Plasmid TAL1/pCMV4 encoding full-length TAL1 polypeptides contains a 1.3 kb *TAL1* cDNA fragment in the *Bgl*III site of pCMV4. Site-directed mutagenesis of TAL1/pCMV4 was performed with the Muta-Gene in vitro mutagenesis kit (Bio-Rad) according to the method of Kunkel et al. (30).

RESULTS

Tryptic maps of TAL1 phosphoproteins. We had previously identified TAL1 phosphoproteins in both erythroleukemia and T-ALL cell lines (18). In either setting, the pp42^{TAL1} polypeptide is phosphorylated at serine but not at threonine or tyrosine residues (18). We initially set out to localize the phosphorylation sites within pp42^{TAL1} and to identify the cellular kinases that catalyze TAL1 phosphorylation. Therefore, pp42^{TAL1} was purified from $^{32}\text{P}_i$ -labeled cell lines by immunoprecipitation and SDS-polyacrylamide gel electrophoresis as described previously (18). Tryptic maps were then generated by digesting purified pp42^{TAL1} with trypsin and separating the hydrolyzed fragments in one dimension by electrophoresis and in a second dimension by ascending chromatography. As illustrated in Fig. 1A, two major ^{32}P -labeled fragments were found in tryptic maps of pp42^{TAL1} from Jurkat T-ALL cells: a well-resolved species that migrates rapidly in both dimensions (phosphopeptide 1) and a poorly resolved species with slow mobilities in both dimensions (phosphopeptide 2). A minor fragment was also apparent upon a longer exposure of the autoradiogram (phosphopeptide 3). As shown in Fig. 1B, the tryptic map of pp42^{TAL1} from the erythroleukemic cell line K562 is similar except that phosphopeptide 3 is more prominent.

Tryptic maps were also prepared from exogenous TAL1 phosphoproteins produced in COS1 cells transfected with recombinant TAL1 expression vectors. Hence, COS1 cells,

FIG. 2. Tryptic maps of pp22^{TAL1} and pp42^{TAL1} from transfected COS1 cells. COS1 cells were transfected with either the TAL1m176/pCMV4 (A) or TAL1/pCMV4 (B) expression vector and labeled with ³²P_i. The TAL1 phosphoproteins pp22^{TAL1} (A) and pp42^{TAL1} (B) were then purified from cell lysates by immunoprecipitation and SDS-polyacrylamide gel electrophoresis. Tryptic digests of these proteins were applied to thin-layer cellulose plates and fractionated in the first dimension by electrophoresis at pH 1.9 (anode to the left) and in the second dimension by ascending chromatography in isobutyric acid buffer. Autoradiograms and schematic diagrams of the tryptic maps are shown. The mobilities of the 2,4-dinitrophenol-lysine (DNP-K) markers in both dimensions are indicated by oval circles.

which do not normally produce TAL1 polypeptides, were transiently transfected with vectors that encode either the full-length pp42^{TAL1} (TAL1 residues 1 to 331) or the truncated pp22^{TAL1} (residues 176 to 331). Tryptic maps of pp42^{TAL1} from transfected COS1 cells are similar to those of Jurkat pp42^{TAL1} with the exception that phosphopeptide 2 resolves into a series of fragments with differing mobilities in the first dimension (phosphopeptides 2a to 2d) (Fig. 2B). As illustrated in Fig. 2A, the truncated pp22^{TAL1} polypeptide from transfected COS1 cells retains phosphopeptides 2a to 2d but does not have phosphopeptide 1. Thus, phosphopeptide 1 is probably derived from the amino-terminal moiety of pp42^{TAL1} (i.e., residues 1 to 176).

FIG. 3. In vitro phosphorylation of GST-TAL1 fusion proteins with ERK1 protein kinase. (A) Schematic diagram illustrating the amino acid composition of the GST-TAL1 fusion proteins used as substrates for in vitro phosphorylation by ERK1 kinase. The GST-TAL1 fusion proteins are composed of GST (closed bars) and segments of the TAL1 polypeptide (open bars). The numbers indicate amino acid residues of TAL1. The ERK1 phosphorylation site at TAL1 residue S122 is indicated by an asterisk. The initiator methionines of TAL1 are indicated (M), and the TAL1 helix-loop-helix (HLH) region is denoted by a black rectangle. (B) Assay in which the GST-TAL1 fusion proteins were phosphorylated in vitro by ERK1 kinase in the presence of [γ -³²P]ATP and fractionated by electrophoresis on an SDS-12.5% polyacrylamide gel. An autoradiogram of the dried gel is shown. Numbers above the lanes denote the TAL1 amino acid residues of the corresponding GST-TAL1 fusion protein. The mobilities of molecular weight markers are indicated in kilodaltons on the left.

In vitro phosphorylation of TAL1 by ERK1 protein kinase.

Because of their critical role in growth factor signal transduction, we sought to determine whether the ERK protein kinases are responsible for TAL1 phosphorylation. Therefore, overlapping segments of the full-length TAL1 polypeptide were isolated in the form of fusion proteins with glutathione S-transferase (GST) (Fig. 3A). Equivalent amounts of each GST-TAL1 fusion polypeptide were incubated with purified ERK1 protein kinase in the presence of [γ -³²P]ATP, and each phosphorylation reaction was then fractionated by SDS-polyacrylamide gel electrophoresis. As shown in Fig. 3B, the only polypeptide to incorporate significant levels of the ³²P label was GST-TAL(1-210), a fusion protein containing the amino-terminal 210 residues of TAL1. Since the other polypeptides, including GST-TAL(1-121) and GST-TAL(166-331), were not effective substrates

FIG. 4. Tryptic map of in vitro-phosphorylated GST-TAL(1-210). The GST-TAL(1-210) fusion protein was phosphorylated in vitro by ERK1 kinase in the presence of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and purified by SDS-polyacrylamide gel electrophoresis as illustrated in Fig. 3. A tryptic digest of phosphorylated GST-TAL(1-210) was applied to a thin-layer cellulose plate and fractionated in the first dimension at pH 1.9 (anode to the left) and in the second dimension by ascending chromatography in isobutyric acid buffer. An autoradiogram and a schematic diagram of the tryptic map are shown. The mobilities of the 2,4-dinitrophenol-lysine (DNP-K) marker in both dimensions are indicated by oval circles.

for ERK1 kinase, a potential ERK1 phosphorylation site is likely to reside within TAL1 amino acids 121 to 166.

To determine whether the in vitro substrate for ERK1 kinase within GST-TAL(1-210) corresponds to a physiologically relevant TAL1 phosphorylation site, tryptic maps were prepared from the in vitro-phosphorylated GST-TAL(1-210) fusion protein. As illustrated in Fig. 4, the major tryptic fragment exhibits mobilities in both dimensions that are indistinguishable from those of phosphopeptide 1 from in vivo-labeled pp42^{TAL1} (cf. Fig. 1 and 2). The identity between these phosphopeptides was further ascertained by a direct comigration experiment. Thus, in vitro-labeled phosphopeptide 1 was recovered from the tryptic map of ERK1-phosphorylated GST-TAL(1-210) (Fig. 4), and in vivo-labeled phosphopeptide 1 was obtained from a tryptic map of pp42^{TAL1} from transfected COS1 cells (Fig. 2B). As demonstrated in Fig. 5, the in vitro- and in vivo-labeled phosphopeptides comigrate precisely upon two-dimensional fractionation on thin-layer cellulose. These data indicate that phosphopeptide 1 of pp42^{TAL1} encompasses an in vitro phosphorylation site for ERK1 kinase and that this site resides within TAL1 amino acids 121 to 166.

Identification of the phosphorylation site within TAL1 phosphopeptide 1. TAL1 amino acids 121 to 166 include five serine residues distributed within two potential tryptic fragments: peptide 118-134 (MVQLSPPALAAPAGR) and peptide 135-166 (ALLYSLSQPLASLGSGFFGEPDAFPMFTTNNR). Therefore, both fragments are candidates for phosphopeptide 1. Three lines of evidence suggest that phosphopeptide 1 is derived from the smaller fragment (tryptic peptide 118-134). First, the extreme hydrophobicity of peptide 118-134 is consistent with the unusually rapid migration of phosphopeptide 1 during ascending chromatography (i.e., the second dimension of the tryptic maps). Second, peptide 118-134, but not peptide 135-166, contains the minimal consensus sequence (SP or TP) currently rec-

FIG. 5. Comigration of in vivo- and in vitro-labeled phosphopeptide 1. In vivo-labeled phosphopeptide 1 was isolated from a tryptic map of pp42^{TAL1} from $^{32}\text{P}_i$ -labeled COS1 cells. In vitro-labeled phosphopeptide 1 was isolated from a tryptic map of ERK1-phosphorylated GST-TAL(1-210) fusion protein. Aliquots of in vivo-labeled phosphopeptide 1 (in vivo), in vitro-labeled phosphopeptide 1 (in vitro), and a mixture of both (in vivo + in vitro) were applied to a thin-layer cellulose plate at distinct positions along the first dimension; each sample origin is indicated by an asterisk. The samples were fractionated in the first dimension by electrophoresis at pH 1.9 (anode to the left) and in the second dimension by ascending chromatography in isobutyric acid buffer, and the phosphopeptides were then detected by autoradiography. The mobility of the 2,4-dinitrophenol-lysine (DNP-K) marker in the second dimension is indicated by an oval circle.

ognized for substrates of ERK1 protein kinase (24). Third, phosphopeptide 1 recovered from tryptic maps of pp42^{TAL1} was completely refractory to digestion with staphylococcal V8 protease (data not shown); peptide 118-134, in contrast to peptide 135-166, does not contain a potential cleavage site for V8 protease, an endopeptidase that hydrolyzes peptide bonds adjacent to aspartate or glutamate residues. In sum, these observations imply that phosphopeptide 1 is derived from tryptic fragment 118-134 and that the serine at residue 122 (S122) is a phosphorylation site for ERK1 protein kinase.

To confirm that phosphopeptide 1 is the phosphorylated derivative of tryptic fragment 118-134, a synthetic peptide was prepared on the basis of the primary amino acid sequence of peptide 118-134. As shown in Fig. 6, the synthetic peptide is a substrate for in vitro phosphorylation by purified ERK1 protein kinase. Moreover, the in vitro-phosphorylated synthetic peptide comigrates precisely with in vivo-labeled phosphopeptide 1 upon two-dimensional fractionation on thin-layer cellulose (Fig. 7).

Final confirmation that S122 is the phosphorylation site of phosphopeptide 1 was obtained by in vitro mutagenesis. Therefore, the TAL1/pCMV4 expression vector that encodes pp42^{TAL1} was altered by site-directed mutagenesis such that the codon specifying S122 (AGT) would instead specify an alanine residue (GCT). COS1 cells were then transfected with the mutant expression vector (TAL1-S122A/pCMV4) and labeled with $^{32}\text{P}_i$. Mutant pp42^{TAL1} was immunoprecipitated from cell lysates with an anti-TAL1 antiserum and purified by SDS-polyacrylamide gel electrophoresis. As illustrated in Fig. 8, phosphopeptide 1 is

FIG. 6. In vitro phosphorylation of a synthetic TAL1 peptide by ERK1 protein kinase. A synthetic TAL1 peptide (residues 118 to 134) was used as a substrate for in vitro phosphorylation by ERK1 protein kinase in the presence of [γ - 32 P]ATP. The phosphorylation products were then fractionated on a thin-layer cellulose plate by ascending chromatography in isobutyric acid buffer. An autoradiogram of the chromatogram is shown. The phosphorylation reactions were conducted in the presence (+) or absence (-) of the indicated components. The positions of the phosphorylated peptide and the unincorporated [γ - 32 P]ATP are marked with arrows.

conspicuously absent from the tryptic map of mutant pp42^{TAL1}.

Stimulation of S122 phosphorylation by treatment with EGF. ERK/MAP2 protein kinases such as ERK1 and ERK2

FIG. 7. Comigration of in vivo-labeled phosphopeptide 1 and an in vitro-labeled synthetic peptide. In vivo-labeled phosphopeptide 1 was isolated from a tryptic map of pp42^{TAL1} from 32 P-labeled COS1 cells. In vitro-labeled peptide was produced by phosphorylation of a synthetic TAL1 peptide (residues 118 to 134) by ERK1 protein kinase and isolated by thin-layer chromatography as shown in Fig. 6. Aliquots of in vivo-labeled phosphopeptide 1 (in vivo), in vitro-labeled synthetic peptide (in vitro), and a mixture of both (in vivo + in vitro) were applied to a thin-layer cellulose plate at distinct positions along the first dimension; each sample origin is indicated by an asterisk. The samples were fractionated in the first dimension by electrophoresis at pH 1.9 (anode to the left) and in the second dimension by ascending chromatography in isobutyric acid buffer, and the phosphopeptides were then detected by autoradiography. The mobility of the 2,4-dinitrophenol-lysine (DNP-K) marker in the second dimension is indicated by an oval circle.

FIG. 8. Tryptic map of the mutant pp42^{TAL1-S122A} phosphoprotein. COS1 cells were transfected with expression vector TAL1-S122A/pCMV4 and labeled with 32 P_i. Mutant pp42^{TAL1-S122A} was purified from cell lysates by immunoprecipitation with an anti-TAL1 antiserum and electrophoresis on an SDS-polyacrylamide gel. A tryptic digest of pp42^{TAL1-S122A} was applied to a thin-layer cellulose plate and fractionated in the first dimension by electrophoresis at pH 1.9 (anode on the left) and in the second dimension by ascending chromatography in isobutyric acid buffer. An autoradiogram and a schematic diagram of the tryptic map are shown. The mobilities of the 2,4-dinitrophenol-lysine (DNP-K) marker in both dimensions are indicated by oval circles.

appear to be ubiquitously expressed in the different tissues and cell types of animals (9, 19, 41, 43). Also, specific inhibitors for this class of protein kinases are not yet available. Therefore, it is difficult to obtain definitive evidence that the in vivo phosphorylation of a given protein is in fact mediated by a cellular ERK kinase. Nevertheless, the enzymatic activities of these kinases are dramatically enhanced upon stimulation with a variety of extracellular ligands, including growth factors such as insulin and EGF. Therefore, we sought to determine whether in vivo phosphorylation of TAL1 is increased by treatment of transfected COS1 with EGF. To reduce the ERK1 kinase activity in unstimulated cells, TAL1/pCMV4-transfected COS1 cells were cultured in a serum-free, methionine-free medium for 3 h prior to cell lysis; for the final 2 h, the cells were labeled with [35 S]methionine. Radiolabeled cells were then cultured either in the presence or in the absence of purified EGF for 10 min immediately before cell lysis. TAL1 polypeptides were purified from cell lysates by immunoprecipitation with an anti-TAL1 antiserum, incubated in the presence or absence of bacterial alkaline phosphatase (BAP), and fractionated by SDS-polyacrylamide gel electrophoresis. As illustrated in Fig. 9, two 35 S-labeled TAL1 polypeptides of 41 and 42 kDa were observed. We had previously shown that p41^{TAL1} and pp42^{TAL1} correspond respectively to unphosphorylated and phosphorylated TAL1 gene products (18); these assignments are confirmed by the fact that pp42^{TAL1}, but not p41^{TAL1}, is lost upon incubation of the immunoprecipitates with BAP (Fig. 9). As shown in Fig. 9, the proportion of phosphorylated TAL1 polypeptides (i.e., pp42^{TAL1}) is significantly increased by treatment of the transfected COS1 cells with EGF.

To determine whether EGF treatment specifically induces phosphorylation of the ERK1 site at amino acid residue S122, a similar experiment was conducted except that the

FIG. 9. Effect of EGF stimulation on TAL1 protein phosphorylation. TAL1/pCMV4-transfected COS1 cells were cultured in serum-free media, labeled with [35 S]methionine, and treated either with (+) or without (-) EGF for 10 min before cell lysis. Each cell lysate was immunoprecipitated with an anti-TAL1 antiserum. The immunoprecipitates were incubated in the presence (+) or absence (-) of BAP and then fractionated by electrophoresis on an SDS-8 to 15% polyacrylamide gradient gel. A fluorogram of the dried gel is shown. The mobilities of immunoprecipitated p41^{TAL1} and pp42^{TAL1} are marked on the left.

transfected COS1 cells were instead labeled with 32 P_i. 32 P-labeled pp42^{TAL1} was purified by immunoprecipitation with an anti-TAL1 antiserum and electrophoresis on an SDS-polyacrylamide gel. Tryptic maps of pp42^{TAL1} from EGF-treated and untreated cells were then compared. As illustrated in Fig. 10, the incorporation of 32 P label into phosphopeptide 1 was significantly increased in pp42^{TAL1} from EGF-treated cells.

DISCUSSION

The *TAL1* gene encodes phosphoproteins, pp42^{TAL1} and pp22^{TAL1}, that represent phosphorylated versions of the

FIG. 10. Effect of EGF stimulation on the in vivo phosphorylation of TAL1 residue S122. TAL1/pCMV4-transfected COS1 cells were cultured in serum-free medium, labeled with 32 P_i, and treated with (B) or without (A) epidermal growth factor (EGF) for 10 min before cell lysis. pp42^{TAL1} was purified from each cell lysate by immunoprecipitation and SDS-polyacrylamide gel electrophoresis. Tryptic digests of purified pp42^{TAL1} were applied to thin-layer cellulose plates and fractionated in the first dimension by electrophoresis at pH 1.9 (anode on the left) and in the second dimension by ascending chromatography in isobutyric acid buffer. Autoradiograms of the tryptic maps are shown. The mobilities of the 2,4-dinitrophenol-lysine (DNP-K) markers in both dimensions are indicated by oval circles.

full-length (residues 1 to 331) and truncated (residues 176 to 331) TAL1 polypeptides, respectively (18). The results described above indicate that amino acid S122 is a major phosphorylation site of pp42^{TAL1} in both erythroleukemia and T-ALL cell lines. The effect of phosphorylation at this site on the function of pp42^{TAL1} is not known. Nevertheless, previous studies have shown that protein phosphorylation can radically alter the properties of other transcriptional regulatory factors, including those that are implicated in tumorigenesis (10, 28, 29). For example, the DNA-binding activities of the c-Myb and c-Jun oncoproteins are abrogated by phosphorylation of sites near their DNA recognition domains (14, 31). Alternatively, phosphorylation of specific residues within the transcriptional activation domains of c-Jun and c-Myc has been shown to increase their transactivation potential (8, 36, 39). Within the primary sequence of TAL1, amino acid residue S122 is well separated from the bHLH domain (residues 188 to 243). Thus, it seems unlikely that phosphorylation of S122 would affect either the dimerization of TAL1 with other bHLH proteins (e.g., E12 or E47) or the ability of such heterodimers to recognize DNA. Instead, S122 phosphorylation may influence interactions between TAL1 and other factors, including elements of the transcription initiation complex or intermediary proteins that themselves recognize the initiation complex.

The enzymatic activities of the ERK/MAP2 protein kinases are increased very rapidly by a variety of extracellular stimuli, including growth factors, activators of protein kinase C, and antigen recognition by T lymphocytes (9, 19, 41, 43). ERK activation appears to be a critical component of signal transduction pathways that mediate the cellular response to these stimuli. Although a number of physiological substrates for activated ERK kinases have been proposed, in surprisingly few cases have their phosphorylation sites been defined. Alvarez et al. (1) identified in vitro phosphorylation sites for ERK in the EGF receptor (T669; EPLTPSG) and the c-Myc (S62; PPLSPSR) and c-Jun (S243; PPLSPID) oncoproteins. Significantly, each of these residues is phosphorylated in intact cells, although the responsible in vivo kinase(s) has not been definitively determined (1, 14, 25, 42). On the basis of the primary sequences surrounding these sites, Alvarez et al. (1) proposed a potential consensus sequence (PLSP or PLTP) for substrates of ERK protein kinases. Other sites within c-Jun (S63 and S73) have also been described as ERK substrates by some but not all investigators (1, 36). More recently, Haycock et al. (24) have suggested that a consensus sequence of only SP or TP may be sufficient because residue S31 of rat tyrosine hydroxylase (S31; AVTSPRF) is an excellent substrate for ERK1 and ERK2.

Our data provide direct evidence that an in vivo phosphorylation site of TAL1 (residue S122) also serves as an in vitro substrate for ERK1 protein kinase. The amino acid sequence surrounding this site (VQLSPPA) is similar to the ERK consensus sequence suggested by Alvarez et al. (1) and identical to that suggested by Haycock et al. (24). Moreover, the ability of EGF to augment S122 phosphorylation within 10 min indicates that the responsible in vivo kinase has the rapid activation profile characteristic of ERK protein kinases (9, 19, 41, 43). Thus, it seems reasonable to propose that TAL1 residue S122 is also an in vivo substrate for ERK kinases such as ERK1. At present, we do not know the effect of S122 phosphorylation on the functional properties of TAL1. If that effect is significant, then TAL1 polypeptides may be subject to regulation by a host of different extracellular signals.

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