Mitogen-activated protein kinase mediates erythropoietin-induced phosphorylation of the TAL1/SCL transcription factor in murine proerythroblasts

Tong TANG*, K. S. Srinivasa PRASAD*¹, Mark J. KOURY*^{†‡} and Stephen J. BRANDT*^{†‡§}²

*Department of Medicine, Vanderbilt University Medical Center, Nashville, TN 37232, U.S.A., [†]Vanderbilt-Ingram Cancer Center, Vanderbilt University Medical Center, Nashville, TN 37232, U.S.A., [‡]Department of Cell Biology, Vanderbilt University Medical Center, Nashville, TN 37232, U.S.A., and [§]Department of Veterans Affairs Medical Center, Nashville, TN 37232, U.S.A.

Ectopic expression of the basic helix-loop-helix transcription factor TAL1 (or SCL) is the most frequent gain-of-function mutation in T-cell acute lymphoblastic leukaemia. Gene-knock-out studies in mice have demonstrated that *TAL1* is required for embryonic and adult haematopoiesis, and considerable evidence suggests it also has important functions in terminal erythroid differentiation. We reported previously that TAL1 phosphorylation is stimulated by erythropoietin in splenic proerythroblasts isolated from mice infected with the anaemia-inducing strain of Friend virus and show here the signalling pathway responsible. Erythropoietin was found to stimulate nuclear mitogen-activated protein kinase activity in addition to TAL1 protein phosphorylation, both of which were quantitatively inhibited by the mitogen-activated protein kinase kinase inhibitor PD 098059 and the phosphatidylinositol 3-kinase inhibitor wortmannin. Tryptic phosphopeptide analysis of radiolabelled TAL1 immunoprecipi-

tated from nuclear extracts of Friend virus-induced proerythroblasts revealed that phosphorylation of Ser¹²², shown previously to be a substrate for the mitogen-activated protein kinase ERK1 (extracellular signal-regulated protein kinase) *in vitro*, was specifically, although not exclusively, increased by erythropoietin and inhibited by wortmannin and PD 098059. These results are consistent with an erythropoietin-stimulated signalling pathway in which there is direct activation of a mitogen-activated protein kinase kinase by phosphatidylinositol 3-kinase and identify TAL1 as one of its nuclear targets. These data suggest, in addition, a specific mechanism by which the principal regulator of erythroid differentiation could enhance TAL1 function, in addition to increasing its expression.

Key words: nuclear substrate, protein phosphorylation, signal transduction.

INTRODUCTION

The *TAL1* gene (also known as *SCL* and *TCL5*) was first identified from molecular analysis of the recurrent chromosomal translocation t(1;14)(p32; q11) in T-cell acute lymphoblastic leukaemia (T-ALL; reviewed in [1]). As a result of this translocation, *TAL1* is juxtaposed to the T-cell receptor α/δ locus and expressed in ectopic fashion. An interstitial deletion which removes 90 kb of upstream sequence occurs in another 25% of T-ALL patients [2,3] and leads to fusion of the *TAL1* coding region, usually in intact form, with the promoter of the *SIL* (*SCL*-interrupting locus) gene. In still other patients, the gene is transcribed in the apparent absence of genomic rearrangements [4]. Misexpression of *TAL1* by one of these mechanisms characterizes up to 60% of individuals with T-ALL, making it the most frequent gain-of-function mutation observed in this type of leukaemia. Two related genes similarly identified as a result of their translocation into T-cell receptor loci, *LYL1* [5] and *TAL2* [6], are also misexpressed in this disease, although far less frequently.

The *TAL1*, *TAL2* and *LYL1* genes are members of the helix-loop-helix (HLH) class of transcription factors, so-named for the conformation adopted by its defining motif of two amphipathic

helices with an intervening loop [7]. The HLH domain promotes the formation of protein hetero- and homodimers, while an adjacent region rich in basic amino acids and present in many of the members of this gene family mediates sequence-specific DNA binding. Similar to other tissue-restricted HLH proteins, TAL1 heterodimerizes with the products of more widely expressed basic-HLH genes referred to collectively as E proteins. These complexes recognize a hexanucleotide motif CANNTG, or E box, in the regulatory regions of the genes they control [8,9].

The *TAL1* locus encodes up to three protein products. The largest (pp47^{TAL1}) has been observed in nearly all cells that express the gene [10,11], while a second protein initiating at the next in-frame AUG codon (pp45^{TAL1}) has been demonstrated in programmed reticulocyte lysates, transfected COS cells and certain cell lines [10,12]. Translation of an even more N-terminally truncated protein (pp24^{TAL1}) initiates in what would usually be the second coding exon as a result of alternative splicing. This isoform has been observed in human and murine leukaemia cell lines [10,11] and Friend virus-induced proerythroblasts [13] and constitutes the only TAL1 species expressed in leukaemias with the rare *SIL-TAL1* d³ deletion [14]. All three TAL1 polypeptides contain basic and HLH domains and exhibit sequence-specific DNA-binding activity [9].

Abbreviations used: T-ALL, T-cell acute lymphoblastic leukaemia; HLH, helix-loop-helix; MAPK, mitogen-activated protein kinase; Epo, erythropoietin; FVA, anaemia-inducing strain of Friend erythroleukaemia virus; PI-3K, phosphatidylinositol 3-kinase; FBS, fetal bovine serum; MBP, myelin basic protein; EGF, epidermal growth factor; MEK, mitogen-activated protein kinase kinase; ERK-1, extracellular signal-regulated protein kinase.

¹ Current address: COR Therapeutics, 256 E. Grand Avenue, South San Francisco, CA 94080, U.S.A.

² To whom correspondence should be addressed, at Division of Hematology-Oncology, Room 547 MRB II, Vanderbilt University Medical Center, Nashville, TN 37232, U.S.A. (e-mail stephen.brandt@mcmail.vanderbilt.edu).

The *TAL1* gene products are present in cells as serine phosphoproteins [10,15]. One of two phosphorylation sites so far identified, Ser¹²², was found to serve as a substrate for the mitogen-activated protein kinase (MAPK) ERK-1 (extracellular signal-regulated protein kinase) *in vitro* and in transfected COS cells [16]. This residue is located in a putative transcriptional activation domain situated in the N-terminal portion of the molecule, and its substitution with alanine was found to decrease the transcriptional potency of pp47^{TAL1} by over half [17]. Phosphorylation of a second serine residue, Ser¹⁷², in a conserved region closer to the basic region altered TAL1 DNA-binding activity in a target-dependent manner [18], suggesting that its function is to discriminate between potential DNA-binding sites.

The frequent association between activating mutations in this gene and T-ALL and, more directly, the results of studies in which a *TAL1* transgene was targeted to the thymus in mice [19,20], implicate TAL1 misexpression in leukaemogenesis. In addition to its actions as an oncogene, TAL1 has an indispensable role in haematopoietic differentiation. TAL1 transcripts and protein have been detected in a number of haematopoietic, as well as certain non-haematopoietic, cell types [21–25], and its expression in embryonic development prefigures and parallels sites of blood formation [24]. Further, mouse embryos homozygous for a targeted mutation in *Tall* died in midgestation with the complete absence of yolk-sac blood cells [26,27]. Studies evaluating the ability of *Tall*^{-/-} embryonic stem cells to contribute to different tissues in chimaeric mice found that the gene is also required postnatally for generation of all haematopoietic cell types [28,29].

Considerable data support a specific role for *TAL1* in erythroid differentiation. TAL1 expression was found to increase significantly in murine erythroleukaemia cell lines stimulated to differentiate with chemical inducers [30,31]. Further, enforced expression of a *TAL1* cDNA in murine erythroleukaemia cells induced their differentiation, particularly in combination with DMSO, whereas expression of *Tall* antisense RNA inhibited their DMSO-induced differentiation [31]. Similarly, expression of a full-length *TAL1* cDNA potentiated erythroid differentiation of a human leukaemia cell line [32] and increased erythroid colony formation by normal human bone-marrow cells [33], whereas treatment of haematopoietic progenitors with a *TAL1* antisense oligonucleotide led to a reduction in the number of erythroid colonies [34]. Finally, two growth factors important for erythroid differentiation, erythropoietin (Epo) and stem-cell factor, were both found to induce *TAL1* expression [13,35].

In studies employing proerythroblasts from the spleens of mice injected with the anaemia-inducing strain of Friend erythroleukaemia virus (FVA), we observed that Tal1 protein phosphorylation was an early and prominent response to Epo stimulation [13]. Prompted by reports that Epo stimulates MAPK activity in erythroid cell lines [36–38], we investigated whether it would do so in this well-characterized model of erythroid differentiation, which signalling pathways were involved, and whether TAL1 is a downstream target. We report that Tal1 is a target of a phosphatidylinositol 3-kinase (PI-3K)-activated MAPK signalling pathway, and that Epo stimulation results in the specific, although not exclusive, phosphorylation on a serine residue, Ser¹²², shown previously to be phosphorylated by MAPK *in vitro*.

EXPERIMENTAL PROCEDURES

Cell culture

CD₂F₁ mice 8–12 weeks of age were given 10⁴ focus-forming units of the anaemia-inducing strain of Friend spleen focus-

forming virus by tail-vein injection. Animals were sacrificed 2 weeks later and splenic proerythroblasts purified by sedimentation of cells through a linear gradient of 1–2% BSA at unit gravity [39]. These FVA cells were cultured in minimum essential medium supplemented with 20% fetal bovine serum (FBS). Cells were washed once with ice-cold PBS prior to use. When included, the protein-kinase inhibitors wortmannin and PD 098059 (Calbiochem, La Jolla, CA, U.S.A.) were added to culture at concentrations of 100 nM and 50 μ M, respectively, and recombinant human Epo (Ortho Pharmaceuticals, Raritan, NJ, U.S.A.) was used at 4 units/ml. COS cells were grown in Dulbecco's modified Eagle's medium with 10% FBS. All cultures were incubated in a humidified atmosphere of 95% air/5% CO₂.

Site-directed mutagenesis

A 1.0-kb full-length mouse *Tall* cDNA was subcloned into the *Bam*HI site of vector pALTER-1 (Promega, Madison, WI, U.S.A.), and oligonucleotide-mediated mutagenesis was carried out according to the supplier's instructions and verified by dideoxynucleotide sequencing. The mutated cDNA was released from pALTER-1 and subcloned into the *Bam*HI site of vector pcDNA1 (Invitrogen, Carlsbad, CA, U.S.A.) for COS cell transfection.

Immunoblot analysis

Nuclear extracts of FVA cells were prepared by the method of Dignam et al. [40], fractionated by SDS/PAGE on 10% gels, and electrotransferred to PVDF membranes. Membranes were incubated with a rabbit polyclonal antibody reactive with both ERK1 and ERK2 (Upstate Biotechnology, Lake Placid, NY, U.S.A.), and MAPK proteins were detected using alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin.

MAPK assay

FVA cells were incubated in minimum essential medium containing 20% FBS for 30 min and then cultured in the same medium with 2% FBS for 1.5 h prior to drug treatment. Wortmannin, PD 098059 or vehicle was added to cells and, after an additional 30 min, Epo was added at a final concentration of 4 units/ml for 1 h. MAPK protein was immunoprecipitated from FVA cell nuclear extracts by the method of Chen and Blenis [41] using an antibody reactive with both ERK1 and ERK2 (Upstate Biotechnology). Protein-kinase activity was assayed as described by Robbins et al. [42] using myelin basic protein (MBP) as substrate. Briefly, immunoprecipitated protein was incubated with 0.2 mg/ml MBP and 100 μ M [γ -³²P]ATP (2 μ Ci/reaction) at 30 °C in 75 mM Tris/HCl (pH 8.0)/10 mM MgCl₂ for 30 min. Reactions were terminated by addition of electrophoresis sample buffer and the reaction mixtures fractionated by SDS/PAGE on a 15% gel. The extent of MBP phosphorylation was analysed by autoradiographic analysis and quantified by PhosphorImaging of the dried gel (Molecular Dynamics, Sunnyvale, CA, U.S.A.). The significance of differences in mean nuclear MAPK activity was determined with a two-tailed *t* test.

Metabolic labelling of cells and radioimmunoprecipitation analysis

FVA cells were incubated prior to labelling in phosphate-free minimum essential medium with 2% dialysed FBS at 37 °C for 30 min. Cells were metabolically labelled by addition of 0.9 mCi/ml [³²P]orthophosphate (ICN, Irvine, CA, U.S.A.) for 1.5 h, incubated with wortmannin, PD 098059 or vehicle for an additional 30 min, and treated with 4 units/ml Epo for 1 h.

Nuclei were isolated and extracted with ice-cold radio-immunoprecipitation assay buffer (9.1 mM Na_2HPO_4 , 1.7 mM NaH_2PO_4 , pH 7.4, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1.0% Nonidet P-40) containing 1 mM sodium vanadate, 1 mM PMSF, 10 $\mu\text{g}/\text{ml}$ leupeptin, 10 $\mu\text{g}/\text{ml}$ aprotinin and 10 $\mu\text{g}/\text{ml}$ pepstatin A. Radiolabelled Tal1 was immunoprecipitated from nuclear extracts by incubation with affinity-purified anti-TAL1 antibody (1:1000 dilution) [24] at 4 °C overnight, and antigen-antibody complexes were collected on Protein A-Sepharose beads (Sigma, St. Louis, MO, U.S.A.). Immunoprecipitates were washed four times with this buffer, boiled for 5 min in electrophoresis sample buffer and resolved by SDS/PAGE on a 10% gel. The extent of Tal1 phosphorylation was analysed by autoradiographic analysis and quantified by PhosphorImaging of the dried gel. The significance of differences in mean incorporation of radioactivity was determined with a two-tailed *t* test.

COS cells were transfected by calcium phosphate co-precipitation [43] with a full-length Tal1 cDNA or the Ser¹²² → Ala substitution mutant, both cloned into the eukaryotic expression vector pcDNA1 (Invitrogen), and incubated for an additional 72 h. Cells were radiolabelled as described above, and purified epidermal growth factor (EGF) was added at a final concentration of 10 ng/ml 5 min before harvest. Radiolabelled Tal1 was immunoprecipitated with anti-Tal1 antibody, and immunoprecipitates were fractionated by SDS/PAGE and subjected to two-dimensional phosphopeptide analysis as described below.

Two-dimensional phosphopeptide analysis

After immunoprecipitation and fractionation by SDS/PAGE, radiolabelled Tal1 protein was transferred to either a PVDF or nitrocellulose membrane, eluted and digested exhaustively with trypsin. Proteolytic fragments were separated by two-dimensional phosphopeptide analysis by the method of Boyle et al. [44]. Briefly, tryptic digests were applied to cellulose thin-layer electrophoresis plates (EM Science, Gibbstown, NJ, U.S.A.) and fractionated in one direction by electrophoresis in pH 1.9 buffer at 1000 V for 20 min and in a second dimension by ascending TLC in a solvent containing 39% butanol, 30% pyridine and 6% glacial acetic acid. PhosphorImaging was used to visualize radiolabelled digestion products and quantify the phosphorylation of specific proteolytic fragments.

RESULTS

Epo stimulates MAPK activity in Friend virus-induced erythroid progenitors: inhibition by PD 098059 and wortmannin

Using an *in vitro* kinase assay with MBP as substrate, Epo was found to significantly increase MAPK activity in FVA cell nuclei over a physiological range of concentrations (results not shown). Nuclear MAPK activity, which increased within 15 min and was maximal 1 h after the addition of Epo to culture, was quantitatively inhibited by pretreating cells with the specific MAPK kinase (MEK or MKK) inhibitor PD 098059 [45] (Figure 1). Epo-stimulated MAPK activity was also abrogated by wortmannin at a concentration (100 nM) in which it acts as a specific inhibitor of PI-3K activity [46] (Figure 1). As both ERK1 and ERK2 isoforms of MAPK were identified in these cells by immunoblot analysis (results not shown), this Epo-induced and wortmannin- and PD 098059-inhibited increase in MAPK activity could have reflected changes in the activity of either or both enzymes.

Figure 1 Stimulation of MAPK activity by Epo in Friend virus-induced erythroid progenitors

(A) MAPK activity was assayed using MBP as phosphoacceptor following immunoprecipitation of nuclear extracts of FVA cells incubated with (Epo) or without (Control) 4 units/ml recombinant human Epo with an antibody to MAPK. Cells were pretreated as indicated with 100 nM wortmannin or 50 μM PD 098059. (B) Plot of mean nuclear MAPK activity $\pm$ S.E.M. relative to that in vehicle control from three independent experiments. *, $P < 0.03$.

Epo stimulates Tal1 phosphorylation in Friend virus-induced erythroid progenitors

As reported previously [13], Epo significantly increased Tal1 phosphorylation in FVA cell nuclei (Figure 2). Having established their ability to inhibit Epo-stimulated MAPK activity in these cells (Figure 1), the MEK and PI-3K inhibitors were then tested for their effect on Tal1 phosphorylation. At the concentrations effective in inhibiting MAPK activity, PD 098059 and wortmannin similarly and quantitatively inhibited the Epo-induced increase in Tal1 phosphorylation (Figure 2).

Ser¹²² is a target of Epo-stimulated MAPK activity in Friend virus-induced erythroid progenitors: inhibition by PD 098059 and wortmannin

Tryptic phosphopeptide analysis was carried out on radiolabelled Tal1 protein immunoprecipitated from nuclear extracts of Epo-

Figure 2 Stimulation of Tal1 phosphorylation by Epo in Friend virus-induced erythroid progenitors

(A) Tal1 protein was immunoprecipitated from nuclear extracts of FVA cells incubated for 1 h with (Epo) or without (Control) 4 units/ml recombinant human Epo. Cells were pretreated as indicated with 100 nM wortmannin or 50 μ M PD 098059. (B) Plot of mean radioactivity incorporated $\pm$ S.E.M. relative to that in vehicle control from three independent experiments. *, $P < 0.02$.

treated FVA cells to identify the residues in Tal1 phosphorylated by Epo-stimulated signalling pathways. To detect phosphorylation of Ser¹²², found previously to be a preferred site of phosphorylation by ERK1 *in vitro* [16], studies were first carried out with EGF-stimulated COS cells transfected with a wild-type *Tal1* cDNA and a Ser¹²² $\rightarrow$ Ala substitution mutant. Mutation of Ser¹²² completely inhibited EGF-stimulated Tal1 protein phosphorylation in radioimmunoprecipitation analysis (results not shown) and prevented the radiolabelling of a specific tryptic

Figure 3 Phosphotryptic peptide analysis of Tal1 and identification of a fragment containing Ser¹²²

COS cells transfected with full-length Tal1 cDNA or one containing a Ser¹²² $\rightarrow$ Ala (S122A) substitution were metabolically labelled with [³²P]orthophosphate and treated with 10 ng/ml EGF for 5 min to activate MAPK. Tal1 protein was immunoprecipitated from nuclear extracts with polyclonal anti-Tal1 antibody, resolved using SDS/PAGE (10% gel), transferred to a PVDF membrane and digested exhaustively with trypsin. Radiolabelled proteolytic fragments were then subjected to two-dimensional phosphopeptide analysis and visualized by PhosphorImaging. The major proteolytic fragment containing Ser¹²² is indicated with an arrow (middle panel).

Figure 4 Co-migration of Ser¹²²-containing phosphopeptide from transfected COS cells and Friend virus-induced erythroid progenitors

Tal1-transfected COS cells and FVA cells were metabolically labelled with [³²P]orthophosphate and stimulated with 10 ng/ml EGF for 5 min and 4 units/ml recombinant human Epo for 60 min, respectively. Tal1 protein was immunoprecipitated from nuclear extracts with polyclonal anti-Tal1 antibody, resolved using SDS/PAGE (10% gel; results not shown), transferred to a nitrocellulose membrane and digested exhaustively with trypsin. Radiolabelled proteolytic fragments from the two cell types were adjusted to the same d.p.m. values, subjected to two-dimensional phosphopeptide analysis individually (COS, FVA) and after combining (Mix), and visualized by PhosphorImaging. The major proteolytic fragment containing Ser¹²² is shown by an arrow.

fragment encompassing this site in two-dimensional phosphopeptide analysis (Figure 3), confirming that Ser¹²² is the exclusive target of MAP kinase in EGF-treated, TAL1-transfected COS cells [16]. That the same site was also involved in Epo-stimulated FVA cells was verified by the co-migration of this fragment in two-dimensional analysis of a mixture of tryptic digests prepared independently from each cell type (Figure 4). Phosphopeptide analysis of radiolabelled Tal1 protein from FVA cells then showed that phosphorylation of Ser¹²², while detectable even prior to the addition of Epo, was stimulated significantly by Epo

Figure 5 Stimulation of Tal1 Ser¹²² phosphorylation by Epo in Friend virus-induced erythroid progenitors

(Upper panel) FVA cells metabolically labelled with [³²P]orthophosphate were pretreated with (Epo + Wortmannin) or without (Epo) 100 nM wortmannin for 30 min and stimulated with 4 units/ml recombinant human Epo for 1 h. Control cells (Control) were treated with neither wortmannin nor Epo. Tal1 protein was immunoprecipitated from nuclear extracts with polyclonal anti-Tal1 antibody, resolved on a 10% SDS/polyacrylamide gel (results not shown), transferred to a PVDF membrane and digested exhaustively with trypsin. Radiolabelled proteolytic fragments were then subjected to two-dimensional phosphopeptide analysis and visualized by PhosphorImaging. The major proteolytic fragment containing Ser¹²² is denoted with an arrow. (Lower panel) As for the upper panel except that FVA cells were pretreated with (Epo + PD 098059) or without (Epo) 50 μ M PD 098059 for 30 min and stimulated with 4 units/ml recombinant human Epo for 1 h. Control cells (Control) were treated with neither PD 098059 nor Epo. Tal1 protein was immunoprecipitated from nuclear extracts with polyclonal anti-Tal1 antibody, resolved on a 10% SDS/polyacrylamide gel (results not shown), transferred to a nitrocellulose membrane, and exhaustively digested with trypsin. Otherwise as for the upper panel.

and inhibited quantitatively by wortmannin (Figure 5, upper panel) and PD 098059 (Figure 5, lower panel) pretreatment. However, other tryptic fragments also demonstrated Epo-inducible, wortmannin- and PD 098059-sensitive phosphorylation (Figure 5), demonstrating that sites in addition to Ser¹²², presumably also serines from the results of previous phosphoamino acid analysis [13], were direct or indirect targets of a PI-3K-linked MAPK signalling pathway in FVA cells.

DISCUSSION

Friend virus-induced erythroid progenitors constitute one of the best characterized and most physiological cellular models of terminal erythroid differentiation [39]. In contrast to established erythroleukaemia lines, FVA cells complete a full differentiation programme, including nuclear extrusion and reticulocyte formation, and resemble their normal counterparts in having an absolute requirement for Epo for survival [47]. Tal1 protein phosphorylation was found to be an early response to Epo

stimulation in FVA cells [13], and we show here the signalling pathway responsible.

A widely utilized mechanism by which membrane-receptor engagement is transduced into changes in gene expression involves Raf1-mediated activation of MAPK. For a number of peptide hormones and cytokines, including Epo [36–38], receptor binding induces, in sequence, Shc phosphorylation, membrane translocation of Grb2, Ras-mediated GTP hydrolysis, Raf1 phosphorylation with activation of its serine/threonine protein kinase activity, phosphorylation of MEK and, finally, MEK-stimulated phosphorylation of MAPK. Phosphorylation of MAPK promotes its homodimerization and entry into the nucleus, where it phosphorylates both general and cell-type-specific substrates, including transcription factors. Through use of erythroid progenitors from mice homozygous for a targeted mutation in the Epo receptor, Klingmüller and colleagues recently identified a Raf1-independent signalling pathway in which PI-3K recruited to the Epo receptor activates MEK directly [48]. The similar sensitivity of Epo-stimulated MAPK activity to wortmannin observed in Friend virus-induced proerythroblasts provides independent support for the existence of this pathway, and the quantitative inhibition of Epo-induced Tal1 phosphorylation by wortmannin and PD 098059 demonstrates that TAL1 is one of its physiological targets.

Although the propensity for FVA cells to undergo apoptosis precluded their prolonged incubation without Epo or, as frequently done in studies of MAPK activation, FBS, it was of interest that both nuclear MAPK activity and Tal1 phosphorylation were detectable prior to the addition of Epo. This could have resulted from activation of the Raf1/MAPK pathway by Friend virus envelope protein [49] or by residual growth factors, particularly insulin-like growth factor-1 [50], in serum. In any case, the magnitude of the Epo-stimulated increase in MAPK activity and Tal1 phosphorylation *in vivo* is likely to be significantly higher than indicated by these cell-culture experiments. Whatever the explanation for any Epo-independent component of MAPK activation in FVA cells, it appears to be insufficient for their survival and ultimate differentiation.

Previous work by Baer and colleagues established that TAL1 could be phosphorylated by the MAPK ERK1, both *in vitro* and in EGF-treated, TAL1-transfected COS cells [16]. However, as discussed by Cohen [51], because of the potential for artifact when components of signalling pathways are overexpressed, this type of data, while important, does not prove that TAL1 is a substrate for this kinase *in vivo*. The availability of selective chemical inhibitors, for MEK [45] and stress-activated protein kinase-2 [52] at least, permit verification that the phosphorylation of a candidate substrate is prevented in conjunction with the inhibition of a specific protein-kinase activity. With the use of the highly specific MEK inhibitor PD 098059, we establish that TAL1 is indeed a physiological substrate for MAPK in Epo-stimulated murine proerythroblasts.

Tryptic phosphopeptide analysis identified Ser¹²² as the principal site of Epo-stimulated Tal1 phosphorylation in FVA cells (Figure 5), extending studies showing this residue to be preferentially phosphorylated by ERK1 *in vitro* and in EGF-stimulated COS cells [16]. While its phosphorylation was found in TAL1-transfected cells to be essential for the function of a transactivation domain in which this residue is placed [17], we observed recently that the transcriptional potency of Tal1 could be increased independent of Ser¹²² phosphorylation through recruitment of the co-activator p300 [53]. Moreover, in contrast to EGF-treated COS cells (Figure 3) in which Ser¹²² is the sole target of MAPK phosphorylation, phosphopeptide mapping studies in Epo-stimulated FVA cells indicate that sites in addition

to Ser¹²² (Figure 5) and Ser¹⁷² (results not shown) may be direct or indirect targets of MAPK-mediated phosphorylation. These findings thus underscore the importance of evaluating the phosphorylation of a transcription factor in a cell type in which it is physiologically expressed.

Whereas gene-knockout and -overexpression studies have shown that TAL1 has a role in erythropoiesis in the adult, it is unclear at present if this involves cell proliferation, survival or differentiation. In any case, TAL1 is the target of and its phosphorylation a marker for an Epo-stimulated PI-3K-MAPK pathway in erythroid progenitors.

We thank Stanley Cohen for purified EGF and Steve Sawyer, Maurice Bondurant and Ron Wisdom for helpful discussions. This work was supported in part by United States Public Health Service grant R01 HL49118 (to S.B.) and Merit Review grants from the Department of Veterans Affairs (to S.B. and M.K.).

REFERENCES

- Begley, C. G. and Green, A. R. (1999) *Blood* **93**, 2760–2770.
- Aplan, P. D., Lombardi, D. P., Ginsberg, A. M., Cossman, J., Bertness, V. L. and Kirsch, I. R. (1990) *Science* **250**, 1426–1429.
- Brown, L., Cheng, J.-T., Chen, Q., Siciliano, M. J., Crist, W., Buchanan, G. and Baer, R. (1990) *EMBO J.* **9**, 3343–3351.
- Bash, R. O., Hall, S., Timmons, C. F., Crist, W. M., Amylon, M., Smith, R. G. and Baer, R. (1995) *Blood* **86**, 666–676.
- Mellentin, J. D., Smith, S. D. and Cleary, M. L. (1989) *Cell* **58**, 77–83.
- Xia, Y., Brown, L., Yang, C. Y.-C., Tsan, J. T., Siciliano, M. J., Espinosa, R., LeBeau, M. M. and Baer, R. J. (1991) *Proc. Natl. Acad. Sci. U.S.A.* **88**, 11416–11420.
- Murre, C., McCaw, P. S. and Baltimore, D. (1989) *Cell* **56**, 777–783.
- Hsu, H.-L., Cheng, J.-T., Chen, Q. and Baer, R. (1991) *Mol. Cell. Biol.* **11**, 3037–3042.
- Hsu, H.-L., Huang, L., Tsan, J. T., Funk, W., Wright, W. E., Hu, J.-S., Kingston, R. E. and Baer, R. (1994) *Mol. Cell. Biol.* **14**, 1256–1265.
- Cheng, J. T., Hsu, H. L., Hwang, L. Y. and Baer, R. (1993) *Oncogene* **8**, 677–683.
- Hsu, H.-L., Wadman, I. and Baer, R. (1994) *Proc. Natl. Acad. Sci. U.S.A.* **91**, 3181–3185.
- Bernard, O., Lecoindre, N., Jonveaux, P., Souyri, M., Mauchauffe, M., Berger, R., Larsen, C. J. and Mathieu-Mahul, D. (1991) *Oncogene* **6**, 1477–1488.
- Prasad, K. S. S., Jordan, J. E., Koury, M. J., Bondurant, M. C. and Brandt, S. J. (1995) *J. Biol. Chem.* **270**, 11603–11611.
- Bash, R. O., Crist, W. M., Shuster, J. J., Link, M. P., Amylon, M., Pullen, J., Carroll, A. J., Buchanan, G. R., Smith, R. G. and Baer, R. (1993) *Blood* **81**, 2110–2117.
- Goldfarb, A. N., Goueli, S., Mickelson, D. and Greenberg, J. M. (1992) *Blood* **80**, 2858–2866.
- Cheng, J.-T., Cobb, M. H. and Baer, R. (1993) *Mol. Cell. Biol.* **13**, 801–808.
- Wadman, I. A., Hsu, H.-L., Cobb, M. H. and Baer, R. (1994) *Oncogene* **9**, 3713–3716.
- Prasad, K. S. S. and Brandt, S. J. (1997) *J. Biol. Chem.* **272**, 11457–11462.
- Kelliher, M. A., Seldin, D. C. and Leder, P. (1996) *EMBO J.* **15**, 5160–5166.
- Condorelli, G. L., Facchiano, F., Valtieri, M., Proietti, E., Vitelli, L., Lulli, V., Huebner, K., Peschle, C. and Croce, C. M. (1996) *Cancer Res.* **56**, 5113–5119.
- Begley, C. G., Aplan, P. D., Denning, S. M., Haynes, B. F., Waldmann, T. A. and Kirsch, I. R. (1989) *Proc. Natl. Acad. Sci. U.S.A.* **86**, 10128–10132.
- Green, A. R., Lints, T., Visvader, J., Harvey, R. and Begley, C. G. (1992) *Oncogene* **7**, 653–660.
- Hwang, L.-Y., Siegelman, M., Davis, L., Oppenheimer-Marks, N. and Baer, R. (1993) *Oncogene* **8**, 3043–3046.
- Kallianpur, A. R., Jordan, J. E. and Brandt, S. J. (1994) *Blood* **83**, 1200–1208.
- Pulphord, K., Lecoindre, N., Leroy-Viard, K., Jones, M., Mathieu-Mahul, D. and Mason, D. Y. (1995) *Blood* **85**, 675–684.
- Shivdasani, R. A., Mayer, E. L. and Orkin, S. H. (1995) *Nature (London)* **373**, 432–434.
- Robb, L., Lyons, I., Li, R., Hartley, L., Köntgen, F., Harvey, R. P., Metcalf, D. and Begley, C. G. (1995) *Proc. Natl. Acad. Sci. U.S.A.* **92**, 7075–7079.
- Porcher, C., Swat, W., Rockwell, K., Fujiwara, Y., Alt, F. W. and Orkin, S. H. (1996) *Cell* **86**, 47–57.
- Robb, L., Elwood, N. J., Elefanti, A. G., Köntgen, F., Li, R., Barnett, L. D. and Begley, C. G. (1996) *EMBO J.* **15**, 4123–4129.
- Green, A. R., Salvaris, E. and Begley, C. G. (1991) *Oncogene* **6**, 475–479.
- Aplan, P. D., Nakahara, K., Orkin, S. H. and Kirsch, I. R. (1992) *EMBO J.* **11**, 4073–4081.
- Hoang, T., Paradis, E., Brady, G., Billia, F., Nakahara, K., Iscove, N. N. and Kirsch, I. R. (1996) *Blood* **87**, 102–111.
- Elwood, N. J., Zogos, H., Pereira, D. S., Dick, J. E. and Begley, C. G. (1998) *Blood* **91**, 3756–3765.
- Condorelli, G., Vitelli, L., Valtieri, M., Marta, I., Montesoro, E., Lulli, V., Baer, R. and Peschle, C. (1995) *Blood* **86**, 164–175.
- Miller, B. A., Floros, J., Cheung, J. Y., Wojchowski, D. M., Bell, L., Begley, C. G., Elwood, N. J., Kreider, J. and Christian, C. (1994) *Blood* **84**, 2971–2976.
- Todokoro, K., Sugiyama, M., Nishida, E. and Nakaya, K. (1994) *Biochem. Biophys. Res. Commun.* **203**, 1912–1919.
- Miura, Y., Miura, O., Ihle, J. N. and Aoki, N. (1994) *J. Biol. Chem.* **269**, 29962–29969.
- Gobert, S., Duprez, V., Lacombe, C., Gisselbrecht, S. and Mayeux, P. (1995) *Eur. J. Biochem.* **234**, 75–83.
- Sawyer, S. T., Koury, M. J. and Bondurant, M. C. (1987) *Methods Enzymol.* **147**, 340–352.
- Dignam, J. D., Lebovitz, R. M. and Roeder, R. G. (1983) *Nucleic Acids Res.* **11**, 1475–1489.
- Chen, R. H. and Blenis, J. (1990) *Mol. Cell. Biol.* **10**, 3204–3215.
- Robbins, D. J., Cheng, M., Zhen, E., Vanderbilt, C. A., Feig, L. A. and Cobb, M. H. (1992) *Proc. Natl. Acad. Sci. U.S.A.* **89**, 6924–6928.
- Graham, F. L. and van der Eb, A. J. (1973) *Virology* **52**, 456–467.
- Boyle, W. J., van der Geer, P. and Hunter, T. (1991) *Methods Enzymol.* **201**, 110–149.
- Alessi, D. R., Cuenda, A., Cohen, P., Dudley, D. T. and Saltiel, A. R. (1995) *J. Biol. Chem.* **270**, 27489–27494.
- Arcaro, A. and Wymann, M. P. (1993) *Biochem. J.* **296**, 297–301.
- Koury, M. J. and Bondurant, M. C. (1990) *Science* **248**, 378–381.
- Klingmüller, U., Wu, H., Hsiao, J. G., Toker, A., Duckworth, B. C., Cantley, L. C. and Lodish, H. F. (1997) *Proc. Natl. Acad. Sci. U.S.A.* **94**, 3016–3021.
- Muszynski, K. W., Ohashi, T., Hanson, C. and Ruscetti, S. K. (1998) *J. Virol.* **72**, 919–925.
- Damen, J. E., Kros, J., Morrison, D., Pelech, S. and Krystal, G. (1998) *Blood* **92**, 425–433.
- Cohen, P. (1997) *Trends Cell Biol.* **7**, 353–361.
- Cuenda, A., Rouse, J., Doza, Y. N., Meier, R., Cohen, P., Gallagher, T. F., Young, P. R. and Lee, J. C. (1995) *FEBS Lett.* **364**, 229–233.
- Huang, S., Qiu, Y., Stein, R. W. and Brandt, S. J. (1999) *Oncogene*, in the press.

Received 10 May 1999/19 July 1999; accepted 18 August 1999