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Functional divergence of the MAP kinase pathway

ERK1 and ERK2 activate specific transcription factors

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Abstract

Growth factor–receptor interactions at the cell surface eventually leading to the transcriptional activation of immediate early genes is mediated by the mitogen-activated protein kinase (MAP kinase/MAPK) cascade. Here we show that overexpression of extracellular signal-regulated kinase 1 (ERK1) cDNA, encoding $p44^{mapk}$, results in the activation of Elk-1, the serum response factor accessory protein. We also show that overexpression of ERK2, encoding $p42^{mapk}$, activates Myc, but not Elk-1. Therefore, the MAP kinase cascade diverges with at least one specific target for each MAP kinase isoform and provides a novel mechanism for differential regulation of this signaling pathway.

Key words: Elk-1; ERK1; ERK2; MAP kinase; Myc; Signal transduction

1. Introduction

The initiation of mammalian cell proliferation by peptide growth factors involves a programmed series of events that are triggered following ligand–receptor interactions at the cell surface. The transduction of these signals from membrane receptors into the nucleus is mediated by an evolutionarily conserved kinase cascade (reviewed in [1–3]). In these parallel signaling cascades, found in yeast, nematode, *Drosophila*, and mammals, it appears that receptor protein tyrosine kinases activate a common set of molecules that include mitogen-activated protein kinases (MAP kinases)/extracellular signal-regulated kinases (ERKs), MAP kinase/ERK-activating kinase (also referred to as MEK, formerly known as MAP kinase activator), Raf protein kinase, $p21^{ras}$, guanine nucleotide releasing factors, src homology domains-containing adaptors and protein phosphatases (reviewed in [4–15]).

Although many of the biochemical details of the above signaling cascades are still vague, protein phosphorylation by Raf, MEK, and MAP kinases are expected to play significant roles in regulating steps throughout these pathways. Ribosomal protein S6, which may be identical to chromatin-associated pp33 [16], is a downstream target of this signaling cascade. This protein is phosphorylated by ribosomal S6 kinase II (also referred to as $p90^{rsk}$), which in turn is activated after phosphorylation by MAP kinase [17]. MAP kinase now consists of related 40–46 kDa kinases that are activated by a variety of extracellular ligands and can use microtubule-associated protein 2, myelin basic protein, and/or epidermal growth factor receptor as in vitro substrates. Recently, cDNAs encoding rat $p44^{mapk}$ (ERK1) and $p42^{mapk}$ (ERK2), mouse $p44^{mapk}$ and $p42^{mapk}$, human $p40^{mapk}$, $p41^{mapk}$, $p42^{mapk}$ and $p44^{mapk}$ were cloned [18–25]. Amino acid identity between $p44^{mapk}$ and $p42^{mapk}$ is about 78%. Here we report the first functional difference between these two MAP kinases.

In vitro studies with recombinant MAP kinases have shown that they possess a slow intrinsic autophosphorylation activity, and that auto-phosphorylation of tyrosine is accompanied by slow autoactivation [25–28]. This observation has led to a search for a MAP kinase activator which could stimulate MAP kinase autophosphorylation. Recent identification of MEK as a dual-specificity threonine and tyrosine protein kinase for MAP kinases argues against this model [10,29–31]. Although autophosphorylation of MAP kinases may not be a physiological activation mechanism, the overexpression of MAP kinase isoforms, even in the absence of upstream signal input, can help to resolve the function of individual MAP kinase in the signaling cascade.

Efforts to correlate MAP kinase activity with growth factor-regulated gene expression have focused primarily on immediate early genes (reviewed in [32]), which are rapidly and transiently transcribed upon serum stimulation of quiescent fibroblasts in culture. Recent evidence indicates that MAP kinases translocate from the cytoplasm to the nucleus upon serum growth factor stimulation [33,34], consistent with a direct role of MAP kinases in the regulation of transcription factor activity. The serum response element (SRE) in many immediate early gene promoters mediates transcriptional activation in response to serum growth factors [35]. A ternary com-

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plex of serum response factor (SRF) dimer and Ternary complex factor (TCF)/Elk-1 binds to the SRE of the c-*fos* proto-oncogene promoter [36,37]. Recently, TCF/Elk-1 is found to be phosphorylated by MAP kinase [37–40]. Our present study shows that Elk-1 is specifically activated by ERK1 expression. Another well characterized MAP kinase substrate is the product of the c-*myc* proto-oncogene [41–43]. In contrast to Elk-1, c-Myc is specifically activated by ERK2 expression. This identification of specific targets for related protein kinases should lead to a better understanding of how these enzymes are orchestrated within a signaling network as well as provide a clearer picture on the dynamic aspects of cellular regulation in response to external stimuli.

2. Materials and methods

2.1. Plasmids

The *Xma*I–*Eco*RI fragment of pBSERK1 [18] was subcloned into the pMex vector [44] to generate pMex-ERK1. The *Bam*HI–*Eco*RI fragment from pMex-ERK1 was recloned into the pSVneoHMTII vector [45]. The *Eco*RI fragment of pBluescript-ERK1 [23] was subcloned into the pUHD 10–3 vector [46]. Plasmid pCMV-ERK2 was a gift from Dr. Jeng-Horng Her and Dr. Michael Weber. Plasmids pGAL4-ElkC and pGAL4-ElkC A383 A389 mutant were gifts from Dr. Richard Treisman. Plasmids pGAL4-Myc and pGAL4-Myc A62 mutant were gifts from Dr. Roger Davis. Human collagenase promoter plasmid −91/−42 tk CAT4 [47] was a gift from Dr. Hans-Peter Auer and Dr. Peter Herrlich.

2.2. Cell culture

NIH/3T3 fibroblasts (American Type Culture Collection CRL 1658) were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS).

2.3. Transient DNA transfections and chloramphenicol acetyltransferase (CAT) assays

DNA transfections and CAT assays were carried out as described previously [48]. β-Galactosidase activity was used to normalize CAT activity.

2.4. Stable DNA transfections and cadmium chloride induction

DNA transfections by the calcium phosphate precipitation procedure and G418 selection have been described [49]. Pools of stable transfectants were screened for accumulation of ERK1 RNA in response to 5 μM cadmium chloride ($CdCl_2$) in the absence of serum.

2.5. Western blot analysis

NIH/3T3 fibroblasts were harvested 48 h after DNA transfection. Total cellular protein was fractionated by SDS-polyacrylamide gel electrophoresis and then transferred to a PVDF membrane for detection by 12CA5 monoclonal antibody [50], rabbit anti-mouse IgG and [^{125}I]protein A.

3. Results

Overexpression of p44^{erk1} MAP kinase was able to stimulate transcription of the collagenase promoter which contains a single activator protein 1 (AP1) site [51]. Therefore, this kinase may, directly [52,53] or indirectly [54,55], phosphorylate and activate c-Jun which binds the AP1 site. Another known promoter element which is likely downstream of the MAP kinase pathway is the c-*fos* SRE because Raf kinase, acting upstream of MAP kinases [40,56,57], is able to activate promoters

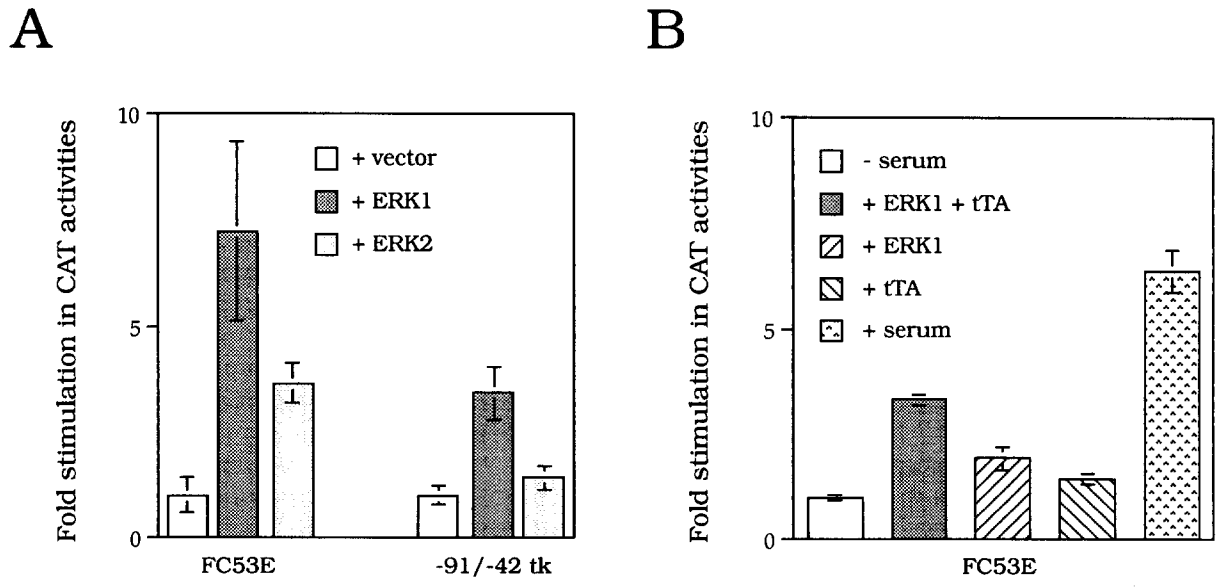

Fig. 1. Constitutive or inducible expression of p44^{erk1} stimulates transcription of the c-*fos* SRE. (Panel A) NIH/3T3 fibroblasts were transfected with 9 μg DNA, including 1.5 μg of a CAT reporter plasmid (pFC53E or p−91/−42 tk CAT4), 0 or 6 μg MAP kinase expression plasmid (pMex-ERK1 or pCMV-ERK2), 0 or 6 μg vector plasmid (pMex), and 1.5 μg β-galactosidase expression plasmid (pCMVβ). Cells were serum-starved after DNA transfection. CAT activities were normalized for differences in transfection efficiency by measurement of the β-galactosidase activity [48]. (Panel B) NIH/3T3 fibroblasts were transfected with 10 μg DNA, including 1 μg of a CAT reporter plasmid (pFC53E), 0 or 2 μg MAP kinase expression plasmid (pUHD 10–3/ERK1), 0 or 2 μg tTA expression plasmid (pUHD15–1), and 1 μg β-galactosidase expression plasmid (pCMVβ). Cells were serum-starved after DNA transfection, and treated with 1 μg/ml tetracycline for 22 h. Cells not transfected with pUHD 10–3/ERK1 and pUHD15–1 were harvested (− serum), or stimulated with FBS (15% final) for 10 h (+ serum). CAT activities were normalized for differences in transfection efficiency by measurement of the β-galactosidase activity [48].

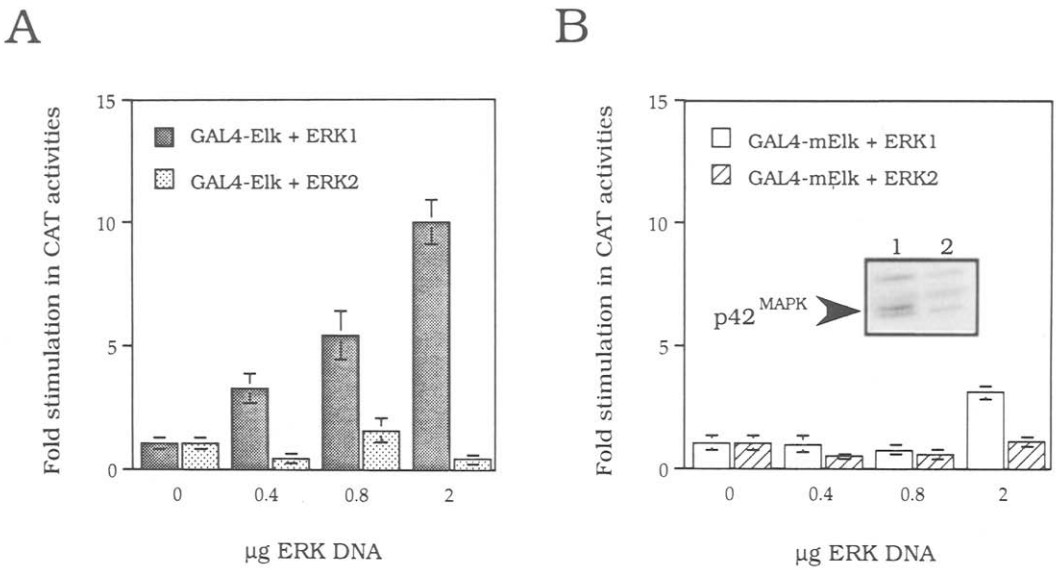

Fig. 2. Expression of $p44^{erk1}$ stimulates the GAL4-ElkC transactivator. (Panel A) NIH/3T3 fibroblasts were transfected with 10 μg DNA, including 1 μg of a CAT reporter plasmid (pG5B CAT), 0.01 μg of pGAL4-ElkC (GAL4-Elk), 0 or 0.4 or 0.8 or 2 μg MAP kinase expression plasmid (pMex-ERK1 or pCMV-ERK2), and 1 μg β-galactosidase expression plasmid (pCMVβ). Cells were serum-starved after DNA transfection. CAT activities were normalized for differences in transfection efficiency by measurement of the β-galactosidase activity [48]. (Panel B) NIH/3T3 fibroblasts were transfected with 10 μg DNA, including 1 μg of a CAT reporter plasmid (pG5B CAT), 0.01 μg of pGAL4-ElkC A383 A389 (GAL4-mElk), 0 or 0.4 or 0.8 or 2 μg MAP kinase expression plasmid (pMex-ERK1 or pCMV-ERK2), and 1 μg β-galactosidase expression plasmid (pCMVβ). Cells were serum-starved after DNA transfection. CAT activities were normalized for differences in transfection efficiency by measurement of the β-galactosidase activity [48]. Inset shows a Western blot of 250 μg protein from NIH/3T3 fibroblasts transfected with pCMV-ERK2 (lane 1) or untransfected (lane 2) fractionated by SDS-PAGE and probed with 12CA5 monoclonal antibody [50]. The arrow indicates the position of 42-kDa MAP kinase.

containing this motif [58]. Furthermore, purified $p44^{erk1}$ MAP kinase phosphorylates the conserved carboxyl-terminal transactivation domain of TCF [38] and Elk-1 [39] in vitro. Here we test whether expression of $p44^{erk1}$ MAP kinase can activate the c-*fos* SRE as well as the Elk-1 transactivation domain in vivo.

To express $p44^{erk1}$ MAP kinase, we constructed the following plasmids: rat cDNA encoding 367 residues of $p44^{erk1}$ [18] was inserted into the pMex vector [44] under the control of the Moloney murine sarcoma virus long terminal repeat (Mo-MSV LTR) for constitutive expression; and the full-length (380 aa) rat ERK1 cDNA [23] was inserted into the pUHD 10–3 vector [46] for tetracycline-inducible expression. As shown in Fig. 1A, cotransfection of pFC53E [59], a c-*fos* SRE reporter plasmid, or human collagenase promoter [47] plasmid, with pMex-ERK1 described above, leads to a 7.2- or 3.4-fold stimulation in CAT activities, respectively. On the other hand, when the tetracycline-inducible $p44^{erk1}$ MAP kinase expression vector was cotransfected with pFC53E and pUHD15–1, encoding tetracycline-controlled transactivator (tTA) [46], CAT activities were stimulated over 3-fold (Fig. 1B).

Next, knowing that, in vitro, $p44^{erk1}$ MAP kinase can phosphorylate Elk-1 [39], which associates with SRF to form the ternary complex over the c-*fos* SRE, we tested whether $p44^{erk1}$ MAP kinase expression can activate the Elk-1 transactivation domain that contains the putative MAP kinase phosphorylation sites [39]. Elk-1 does not bind DNA independently of SRF, therefore we assayed a reporter gene containing GAL4 binding sites, which is recognized by GAL4-Elk-1 fusion proteins.

In quiescent fibroblasts, the activity of the GAL4-Elk-1 fusion protein was stimulated in a dose-dependent manner by increasing amounts of pMex-ERK1 DNA transfected (Fig. 2A). In contrast, similar amounts of pCMV-ERK2 DNA did not stimulate GAL4-Elk-1 activity. Expression of transfected hemagglutinin epitope-tagged ERK2 can be detected with 12CA5 antibody (Fig. 2B, inset), consistent with its functional activity in vivo (see below). Both MAP kinase isoforms can phosphorylate Elk-1 [39,40] but this may be due to a relaxed specificity of MAP kinases in vitro since additional threonine residues are phosphorylated compared with those labeled in vivo [40]. Finally, when the MAP kinase phosphorylation sites (Ser^{383} and Ser^{389}) were mutated to alanines, neither kinase could activate GAL4-Elk-1 (Fig. 2B), showing that these serine residues of Elk-1 are bona fide $p44^{erk1}$ MAP kinase targets.

To confirm the above results, we constructed an ERK1 expression plasmid under the control of the inducible human metallothionein II promoter. Since this plasmid also contains a neomycin phosphotransferase gene, G418-resistant stable transfectants were selected

after DNA transfection into NIH/3T3 fibroblasts. Induction of MAP kinase in these transfectants can lead to activation of the c-*fos* SRE [60]. The basal activity of the GAL4-Elk-1 fusion protein was similar in both ERK1 transfectants and parental NIH/3T3 fibroblasts transfected with pGAL4-ElkC and pG5B CAT (Fig. 3). When ERK1 expression was induced by cadmium chloride, GAL4-Elk-1 activity was stimulated in serum-starved ERK1 transfectant (Fig. 3B), but not in quiescent NIH/3T3 fibroblasts (Fig. 3A), presumably due to higher levels of MAP kinase in the transfectant cells. Endogenous MAP kinases in untransfected NIH/3T3 fibroblasts were activated by serum, not by cadmium chloride. Finally,

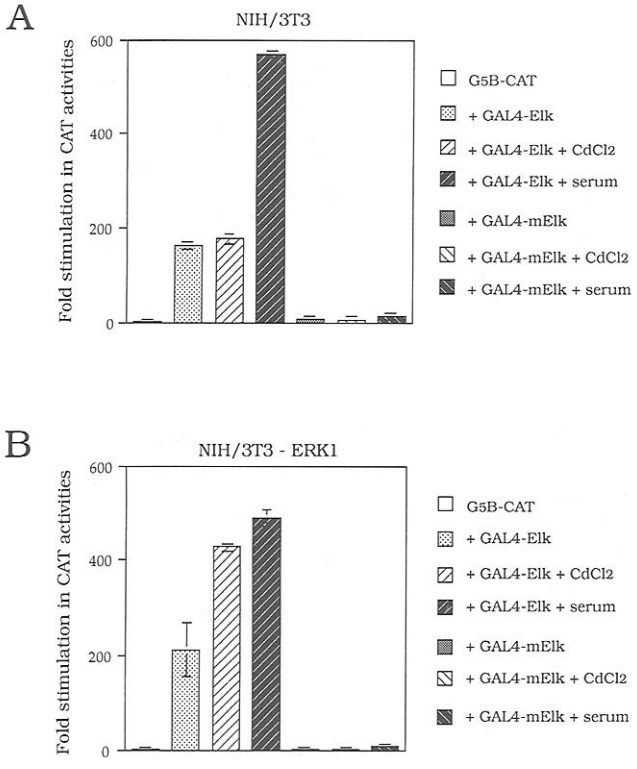

Fig. 3. Inducible expression of $p44^{erk1}$ stimulates the GAL4-ElkC transactivator. (Panel A) NIH/3T3 fibroblasts were transfected with 10 μg DNA, including 1 μg of a CAT reporter plasmid (pG5B CAT), 0.01 μg of pGAL4-ElkC (GAL4-Elk) or pGAL4-ElkC A383 A389 (GAL4- mElk), and 1 μg β-galactosidase expression plasmid (pCMVβ). Cells were serum-starved after DNA transfection. Control induction with metal ion were carried out with cadmium chloride (5 μM) for 16 h. Serum stimulated cells were treated with FBS (15% final) for 10 h. CAT activities were normalized for differences in transfection efficiency by measurement of the β-galactosidase activity [48]. (Panel B) NIH/3T3 fibroblasts stably transfected with pSVneoHMTII-ERK1 were transfected with 10 μg DNA, including 1 μg of a CAT reporter plasmid (pG5B CAT), 0.01 μg of pGAL4-ElkC (GAL4-Elk) or pGAL4-ElkC A383 A389 (GAL4-mElk), and 1 μg β-galactosidase expression plasmid (pCMVβ). Cells were serum-starved after DNA transfection. Human metallothionein II promoter was induced with cadmium chloride (5 μM) for 16 h. Serum stimulated cells were treated with FBS (15% final) for 10 h. CAT activities were normalized for differences in transfection efficiency by measurement of the β-galactosidase activity [48].

GAL4-Elk-1 without the ERK1 phosphorylation sites (as defined above) cannot be activated by cadmium chloride or serum in either cell line.

The novel result that is presented above is that ERK2 expression failed to activate GAL4-Elk-1. This result suggests that $p44^{erk1}$ and $p42^{erk2}$ MAP kinases have specific target substrates. To identify a specific downstream effector for $p42^{erk2}$ MAP kinase, we tested whether only the latter can activate c-Myc. Seth and coworkers [43] have demonstrated that $p42^{erk2}$ MAP kinase expression can activate GAL4-Myc but they did not test the latter in response to $p44^{erk1}$ MAP kinase expression. We also showed that ERK2 expression can induce GAL4-Myc activity in quiescent fibroblasts (Fig. 4A). However, ERK1 expression, either under the control of the constitutive Mo-MSV LTR or the tetracycline-inducible promoter of pUHD 10–3, could not stimulate GAL4-Myc activity (Fig. 4B). Finally, mutation of the Myc MAP kinase phosphorylation site (Ser^{62}) to alanine results in a loss of its ability to be activated by $p42^{erk2}$ MAP kinase. The above results demonstrate that the two MAP kinases have specific downstream targets.

4. Discussion

Based on the conservation of the MAP kinase signaling cascade throughout metazoan evolution, this pathway is important for the regulation of gene expression in response to many extracellular signals. However, the downstream events occurring after MAP kinase activation are far from clear. In order to achieve specificity in signal transduction, there are two main alternative regulatory mechanisms: either functionally redundant MAP kinases act at a branch point in the kinase cascade to phosphorylate multiple proteins in response to both proliferation and differentiation signals; or the pathway actually bifurcates after MEK so that individual MAP kinase targets different transcription factor and/or kinase substrate(s). Our data favors the latter possibility since we have identified specific downstream targets for $p44^{erk1}$ and $p42^{erk2}$ MAP kinases. Nonetheless, our present data do not exclude the possibility that they can share common targets, e.g. $p90^{rsk}$.

Our results indicate that $p44^{erk1}$ and $p42^{erk2}$ MAP kinases are not functionally redundant. We demonstrate here, for the first time, that $p44^{erk1}$ MAP kinase specifically activates the SRF-associated transcription factor Elk-1, and $p42^{erk2}$ MAP kinase specifically activates the immediate early gene product c-Myc. Proliferation and differentiation signals trigger intracellular effectors that converge on the MAP kinase cascade. Then $p44^{erk1}$, $p42^{erk2}$, and $p46^{jnk}$ [55] appears to act at a branch point in the signaling cascade to phosphorylate and activate specific transactivators. Such target specificities of MAP kinases will provide multiple sites for regulation inputs

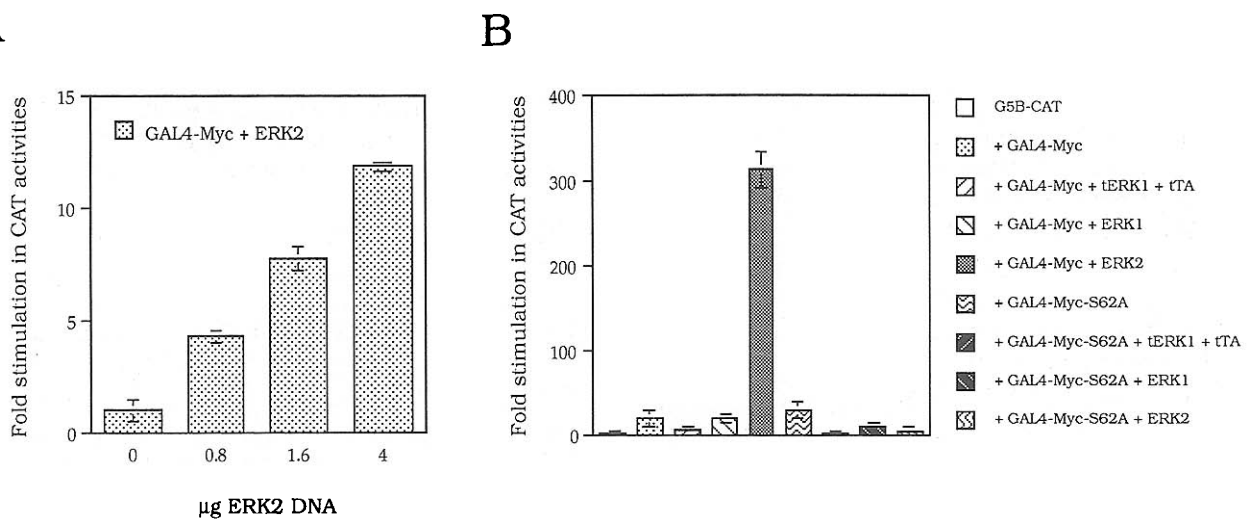

Fig. 4. Expression of $p44^{erk1}$ does not stimulate the GAL4-Myc transactivator. (Panel A) NIH/3T3 fibroblasts were transfected with 10 μg DNA, including 1 μg of a CAT reporter plasmid (pG5B CAT), 0.02 μg of pGAL4/Myc (GAL4-Myc), 0 or 0.8 or 1.6 or 4 μg MAP kinase expression plasmid (pCMV-ERK2), and 1 μg β-galactosidase expression plasmid (pCMVβ). Cells were serum-starved after DNA transfection. CAT activities were normalized for differences in transfection efficiency by measurement of the β-galactosidase activity [48]. (Panel B) NIH/3T3 fibroblasts were transfected with 10 μg DNA, including 1 μg of a CAT reporter plasmid (pG5B CAT), 0.02 μg of pGAL4/Myc (GAL4-Myc) or pGAL4/[Ala^{62}]Myc (GAL4-Myc-S62A), 0 or 2 μg MAP kinase expression plasmid (pMex-ERK1 or pCMV-ERK2) or 2 μg MAP kinase expression plasmid pUHD 10–3/ERK1 (tERK1) and 2 μg tTA expression plasmid (pUHD15–1), and 1 μg β-galactosidase expression plasmid (pCMVβ). Cells were serum-starved after DNA transfection. CAT activities were normalized for differences in transfection efficiency by measurement of the β-galactosidase activity [48].

(e.g. MEK versus protein kinase C) and signal integration (e.g. growth stimuli versus differentiation signals).

In the budding yeast *Saccharomyces cerevisiae*, genetic analyses of the mating response pathway predict an ordered cascade of five kinases: STE20, STE11, STE7, FUS3 and KSS1 (reviewed in [61]). The MAP kinase homologues FUS3 and KSS1 act upstream of the transcription factor STE12. However, FUS3 and KSS1 differ in that only FUS3 is required for cell cycle arrest [62]. Furthermore, FAR1 protein is phosphorylated in response to α factor in *kss1*$^-$ mutant, but not in *fus3*$^-$, *fus3*$^-$ *kss1*$^-$, or *ste7*$^-$ mutant [63]. The above observations indicate that FUS3, or alternatively, a kinase dependent on FUS3, mediates FAR1 phosphorylation [63,64]. Therefore, FUS3 and KSS1 may have both overlapping (e.g. phosphorylation of STE12) and unique (e.g. phosphorylation of FAR1 by FUS3) functions. As the yeast and mammalian kinase cascades are conserved in the order and sequence of the proteins, the regulatory mechanisms leading to the activation of their targets may also be similar. Consistent with the latter idea is our observation that ERK1 and ERK2 have unique functions.

What are the structural determinants of MAP kinase substrate specificity? We noted that the MAP kinase phosphorylation site of Elk-1 (TLSP) belongs to the XX(S/T)P class, whereas the phosphorylation site of c-Myc (PLSP) has a PX(S/T)P motif [11]. If PX(S/T)P sites are phosphorylated specifically by $p42^{erk2}$ MAP kinase, then $p90^{rsk}$ may be another $p42^{erk2}$ MAP kinase target. On the other hand, Fos [65] and Jun [53], with XX(S/T)P sites, appear to be $p44^{erk1}$ MAP kinase substrates. This hypothesis is being tested with GAL4-Jun and GAL4-[$Leu^{63,73}$]Jun plasmids [66].

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