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The kinase suppressor of Ras (KSR) modulates growth factor and Ras signaling by uncoupling Elk-1 phosphorylation from MAP kinase activation

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The Ras GTPase plays an essential role in many cellular signal transduction events. Activation of the mitogen activated protein (MAP) kinase is a primary consequence of Ras activation and plays a key role in mediating Ras signal transduction. A novel kinase, KSR, has recently been functionally isolated as a positive regulator of Ras signaling in *Caenorhabditis elegans* vulval induction and *Drosophila* photoreceptor differentiation. We have examined the effect of KSR on growth factor and Ras-induced MAP kinase signaling in mammalian cells. Surprisingly, we observed that KSR specifically blocks EGF and Ras-induced phosphorylation and activation of ternary complex factors (TCF), physiological substrates of MAP kinases, without affecting the activation of MAP kinase itself. A kinase-deficient mutant of KSR, KSR-RM, appears to function as a dominant interfering mutant which elevates phosphorylation of Elk-1, a member of the TCF family, and Elk-1-dependent transcription. The effect of KSR on Elk-1 was significantly decreased by inhibition of calcineurin, a putative Elk-1 phosphatase. These observations demonstrate that KSR is capable of uncoupling the MAP kinase activation from its target phosphorylation, and thus provide a novel mechanism for modulating the Ras–MAP kinase signaling pathway. This study provides the first evidence that signal output of MAP kinase cascades is subject to regulation at a level independent of MAP kinase activity.

Keywords: Elk-1/KSR/MAP kinase/phosphorylation/Ras

Introduction

Activation of the Ras-mitogen activated protein kinase (MAP kinase, also known as extracellular signal regulated kinase, ERK) cascade is essential for numerous signal transduction pathways including tyrosine kinase receptors, trimeric G-protein-coupled receptors and tyrosine kinase-associated receptors (reviewed in Marshall, 1995; Denhardt, 1996; Robinson and Cobb, 1997). The best understood system is the activation of Ras by receptor tyrosine kinases (van der Geer *et al.*, 1994; Katz and McCormick, 1997). Binding of mitogenic growth factors to its tyrosine kinase receptors results in the activation of Ras. Activated Ras has an essential role in the activation of Raf, which directly phosphorylates and activates MEK, also known as MAP kinase kinase. The activated MEK, in turn, phosphorylates ERK on threonine and tyrosine residues and results in a dramatic activation of ERK. This MAP kinase cascade has been highly conserved throughout evolution and is found in all eukaryotes, including yeast, *Caenorhabditis elegans*, *Drosophila* and mammals (Marshall, 1995). In addition, several MAP kinase related kinases have been discovered. For example, JNK (c-Jun N-terminal kinase, also known as SAPK for stress activated protein kinase) has been found to respond to various stress conditions and phosphorylates and activates c-jun (Hibi *et al.*, 1993; Derijard *et al.*, 1994; Kyriakis *et al.*, 1994). Another well-characterized cascade is the p38 kinase, which is activated by various cytokines and other stimuli (Han *et al.*, 1994; Lin *et al.*, 1995). Both JNK and p38 kinases share a high degree of structural similarity as well as a biochemical mechanism of activation that closely resembles the Raf–MEK–ERK pathway, although different MAP kinase cascades are activated by a set of overlapping and distinct extracellular stimuli.

MAP kinase pathways are involved in numerous developmental programs in model organisms such as *C.elegans* and *Drosophila*. Genetic studies in *C.elegans* and *Drosophila* have identified a novel protein kinase, KSR (kinase suppressor of Ras) as having a positive role in mediating Ras signaling (Kornfeld *et al.*, 1995; Sundaram and Han, 1995; Therrien *et al.*, 1995). Mammalian homologs of KSR have also been identified. Mutations of KSR can suppress the phenotype of a constitutively active mutant of Ras, suggesting that KSR functions downstream of, or parallel to Ras. Mutations in KSR combined with a weak loss-of-function allele of Raf result in a synthetic phenotype, indicating that KSR may function parallel to Raf. Genetic data have established a role of KSR in Ras–MAP kinase signaling, however, the exact function of KSR in the pathway is not clear. The deduced amino acid sequence indicates that KSR is related to the Raf family of protein kinases. Recently, investigators have reported that KSR may indirectly modulate MAP kinase activation in *Xenopus* oocytes (Therrien *et al.*, 1996) while others showed activation of KSR by ceramide (Zhang *et al.*, 1997).

Identification of ERK substrates has provided critical information for understanding the signal transduction of the MAP kinase cascade. Activation of Ras–MAP kinase is known to be essential for the expression of many growth factor-inducible genes. One of the best characterized ERK substrates is Elk-1, which is a member of the TCF (ternary complex factor) family (Gille *et al.*, 1992, 1995; Janknecht *et al.*, 1993; Marias *et al.*, 1993; Kortenjann *et al.*, 1994). Elk-1 belongs to a large family of Ets domain-containing

transcription factors. Members of the Ets family are thought to be involved in regulation of cell transformation and differentiation. Elk-1 contains an N-terminal Ets-like DNA binding domain and a C-terminal ERK responsive transactivation domain. Phosphorylation by activated ERK increases transcriptional activity of Elk-1. This event appears to be critical for ERK-mediated transcription stimulation, as mutation of these phosphorylation sites severely diminishes Elk-1 activity. The C-terminal domain of Elk-1 contains multiple ERK phosphorylation sites. It has been shown that phosphorylation of serine 383 of Elk-1 is critical for the transcriptional activation of Elk-1 (reviewed in Hill and Treisman, 1995). Serine 383 is conserved in two other members of the TCF family, SAP1 and SAP2. Similarly, phosphorylation of these corresponding serine residues is essential for the transcription activity of SAP1 and SAP2 (Giovane *et al.*, 1994; Janknecht and Hunter, 1997). In addition to ERK, both JNK and p38 have been implicated to have a role in regulation of the TCF family members in response to different extracellular signals (Cavigelli *et al.*, 1995; Janknecht and Hunter, 1997; Whitmarsh *et al.*, 1997).

Most of the studies pertaining to MAP kinase signal transduction have identified components directly activating or inactivating the ERK pathway. In fact, a linear pathway from Ras activation to Elk-1 phosphorylation has been established. We have examined the function of KSR on activation and phosphorylation of ERK and Elk-1. Our data show that KSR has no detectable effect on the activity of ERK but blocks growth factor and Ras-induced Elk-1 phosphorylation, which is absolutely ERK-dependent. Similarly, KSR blocks EGF-induced Elk-1 activity. This effect of KSR on Elk-1 appears to require the integrity of its kinase domain since a mutant, KSR-RM, does not inhibit Elk-1. In fact, KSR-RM enhances both Elk-1 phosphorylation and transactivation activity in unstimulated cells. Our results provide a novel function for KSR in modulating the Ras–MAP kinase signal transduction by specifically affecting the activation of Elk-1.

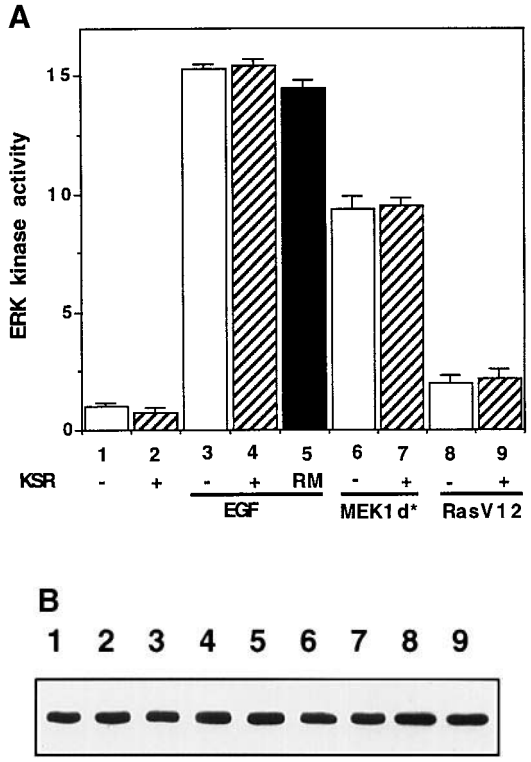

Fig. 1. KSR does not alter the activation of ERK1. (**A**) Effect of KSR on ERK1 activity. COS1 cells in 6 cm dishes were transfected with 0.25 μg of HA-ERK1 (pJH-ERK1; Sells and Chernoff, 1995) in the presence or absence of 1 μg pcDNA3-KSR as indicated. RM denotes the kinase-deficient mutant of KSR. Cells were left untreated or treated with EGF (50 ng/ml, 5 min) as indicated. Co-transfection with either 0.5 μg of RasV12 or MEK1d* (a constitutively active MEK1 mutant) were not treated with EGF. HA-ERK1 was immunoprecipitated with anti-HA antibody and assayed for kinase activity using myelin basic protein as a substrate. The same EGF concentration was used for all the experiments in this report. Results presented (Figures 1–6) are a representative of at least three independent experiments. (**B**) Western blot of immunoprecipitated HA-ERK1. Lanes are as stated in (A). Anti-ERK antibody was used to detect the HA-ERK1 precipitated by anti-HA.

Results

KSR expression does not affect MAP kinase activation in mammalian cells

Previous studies suggest that KSR could act either in parallel to, or on, the Raf–MEK–ERK kinase cascade. Studies in *Xenopus* oocytes have indicated that KSR may indirectly potentiate ERK activation while other experiments suggest that KSR may activate Raf (Therrien *et al.*, 1996; Zhang *et al.*, 1997). We performed experiments to investigate whether KSR can activate the MAP kinase cascade in cultured mammalian cells. Hemagglutinin antigen (HA)-tagged ERK1 was co-transfected into COS1 cells. Transfected cells were rendered quiescent by incubating in low serum medium followed by stimulation with EGF. Co-expression of KSR did not effect basal or stimulated ERK1 activity (Figure 1A and B). Similarly, KSR did not noticeably alter the basal or EGF-stimulated activity of co-transfected MEK1 as determined by an immune-complex kinase assay (data not shown). In contrast, co-expression of Ras or a constitutively active MEK1 mutant significantly activated ERK1.

Activation of ERK1 by co-transfection with Ras or constitutively active MEK1 mutant was also not affected by co-transfection of KSR (Figure 1A and B). Activation of ERK1 requires phosphorylation of two conserved threonine and tyrosine residues. Western blots performed with a phosphorylation state-specific anti-ERK antibody confirmed the results of the kinase activity assays. KSR expression had no detectable effect on ERK phosphorylation (Figure 5A, panel pERK). Identical results were obtained if PMA was used to stimulate MAP kinase activity instead of EGF (data not shown). These results suggest that KSR plays no direct role in either activation or inactivation of MEK–ERK.

KSR blocks transcriptional activation of Elk-1

Activated MAP kinase is able to phosphorylate numerous cellular proteins and thus directly regulate their functions. One ERK target is the TCF, which cooperates with the serum response factor (SRF) to regulate transcription of promoters containing the serum response element (SRE) (Gille *et al.*, 1992, 1995; Janknecht *et al.*, 1993; Marias *et al.*, 1993; Kortenjann *et al.*, 1994). To test the effect of KSR on Elk-1 transcription activity, the activity of a

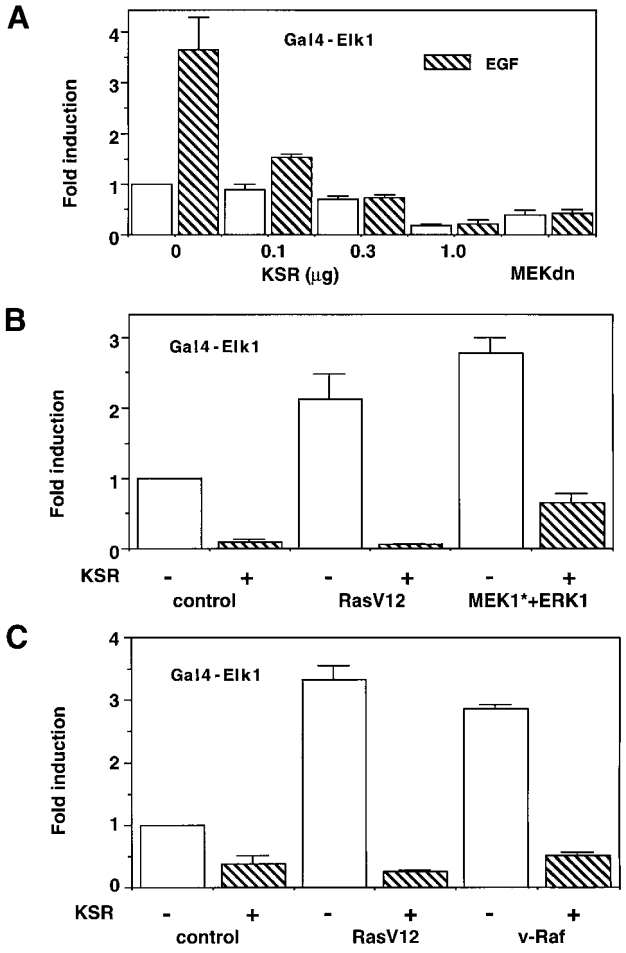

Fig. 2. KSR blocks Elk-1-dependent transcription activity. (**A**) KSR inhibits EGF-induced Gal4–Elk-1 reporter expression. COS1 cells were transfected with 0.2 μg of Gal4–LUC and 0.05 μg of Gal4–Elk-1 together with increasing amounts of pcDNA3-HA-KSR. MEKdn denotes 1.0 μg of the dominant-negative MEK1 plasmid. A pCMV-lacZ plasmid (0.1 μg) was co-transfected as an internal control for variations in transfection efficiency. The transfected cells were left untreated (open bars) or treated (hatched bars) with EGF for 8 h. Luciferase activity was measured and normalized against the co-transfected β-galactosidase activity. (**B**) KSR inhibits Ras and MEK1-induced Gal4–Elk-1 reporter activity. Gal4–Elk-1 reporter was co-transfected with RasV12 (0.5 μg) or constitutively active MEK1* (0.1 μg) together with ERK1 (0.1 μg) into COS1 cells. The presence of KSR (1.0 μg) is indicated. (**C**) Effect of KSR on Gal4–Elk-1 reporter activity in NIH-3T3 cells. NIH-3T3 cells were co-transfected with RasV12 or v-Raf together with Gal4–Elk-1 and Gal4-LUC as above. 24 h post-transfection, cells were incubated in low serum media for additional 24 h and luciferase activity was determined as above. Error bars indicate SD from experiments performed in triplicate. Shown is a representative example of at least three independent experiments.

Gal4–Elk-1 reporter (Marias *et al.*, 1993), which contains the C-terminal ERK responsive domain of Elk-1 fused to the DNA binding domain of Gal4, was tested. The transcription activity of Gal4–Elk-1 is dependent on phosphorylation of the Elk-1 trans-activation domain by ERK. We observed that KSR co-transfection effectively blocked both basal and EGF-stimulated expression of the reporter in a dose dependent manner (Figure 2A). Both untagged and HA-tagged KSR showed identical inhibition of the Gal4–Elk-1 reporter (data not shown). A dominant-negative MEK1 mutant, which has the activation phosphorylation serine residues 218 and 222 replaced by alanine residues, effectively blocked Gal4–Elk-1 activation as expected (Figure 2A), indicating that ERK activation is indeed required for EGF-induced Gal4–Elk-1 activation.

The epistatic relationship between KSR and other components in the Ras–MAP kinase pathway was examined with respect to Gal4–Elk-1 activity by co-transfection with constitutively active mutants of either Ras, Raf or MEK. We observed that RasV12-stimulated Gal4–Elk-1 reporter activity was completely inhibited by co-transfection with KSR in COS1 cells (Figure 2B), supporting previous reports that KSR functions downstream of Ras (Kornfeld *et al.*, 1995; Sundaram and Han, 1995; Therrien *et al.*, 1995). KSR also blocked constitutively active MEK1-induced Gal4–Elk-1 reporter activation. Transfection of v-Raf in NIH-3T3 cells resulted in a significant enhancement of Gal4–Elk-1 reporter activity. Expression of KSR effectively blocked v-Raf-induced Gal4–Elk-1 activity in NIH-3T3 cells (Figure 2C). Similarly, KSR inhibited RasV12-induced Gal4–Elk-1 activation in NIH-3T3 cells (Figure 2C). These observations indicate that KSR functions at a level distinct from known members of the MAP kinase cascade and that the inhibition of Elk-1 transcription is not cell-type specific.

To test whether the inhibition of KSR on Gal4–Elk-1 is specific to Elk-1 rather than a global inhibition of transcription, we performed similar experiments with the Gal4–Jun reporter (Coso *et al.*, 1995). It has previously been demonstrated the activation of the JNK pathway by RacV12 will activate the Gal4–Jun reporter (Coso *et al.*, 1995; Minden *et al.*, 1995). KSR affected neither basal nor Rac1V12-induced Gal4–Jun reporter activity (Figure 3A). It should be emphasized that the Gal4–Jun reporter and the Gal4–Elk reporter are identical except that the Gal4 DNA-binding domain is fused to a different transactivation domain of either the C-terminal ERK-responsive domain of Elk-1 or the N-terminal JNK-responsive domain of c-Jun (Marias *et al.*, 1993; Lin *et al.*, 1995). These observations clearly demonstrate that the effect of KSR on Elk-1 is specific.

KSR expression blocks induction of a c-fos reporter gene

The inhibition of Gal4–Elk-1 by KSR that we observed might simply be an artifact of an artificial chimera transcription factor. To test the effect of KSR on a physiological promoter using endogenous transcription factors, we examined the c-fos reporter for gene expression. The c-fos promoter contains DNA elements for multiple transcription factors, including a functional serum responsive element (SRE) (Hill and Treisman, 1995). SRF together with TCF is responsible for the transcription activation of the SRE (Hill and Treisman, 1995). KSR expression effectively blocked Ras-induced activation of the reporter (Figure 3B) as well as EGF- and PMA-induced c-fos reporter activity (data not shown). The inability of KSR to block the basal activity of the c-fos reporter is probably due to the fact that multiple transcription elements exist in the c-fos promoter, which may contribute to the basal expression. In agreement with this is our observation that KSR expression has no effect on Gal4–Jun activity, which is a component of the AP-1 transcription factor.

In order to test whether KSR specifically inhibited the

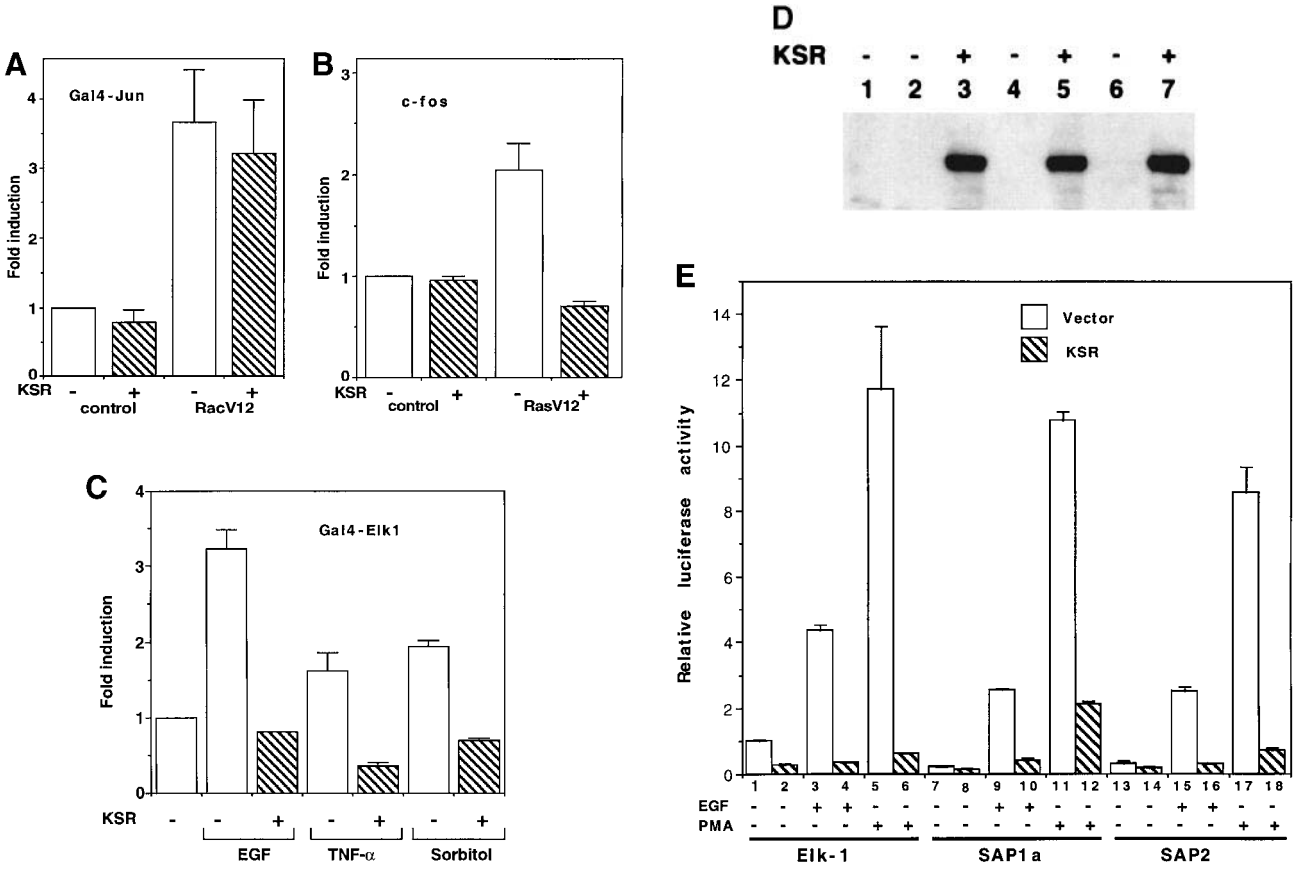

Fig. 3. Effect of KSR on Gal4–Jun and c-fos reporters. (**A**) KSR has no effect on Gal4–Jun activity. Cells were transfected with 0.5 μg of Gal4–LUC, 0.2 μg of Gal4–Jun and 1.0 μg of either pcDNA3, pcDNA3-HA-KSR or a constitutively active mutant of Rac1 (Rac1V12; Minden *et al.*, 1995) as indicated. Reporter activity was measured the same as experiments with Gal4–Elk-1 shown in Figure 2. (**B**) KSR blocks the effect of RasV12 on a c-fos reporter. A luciferase reporter (0.2 μg) controlled by the c-fos promoter (Yamauchi *et al.*, 1993) was co-transfected together with vector control of RasV12 in the presence (hatched bars) or absence (open bars) of KSR. (**C**) KSR blocks TNF-α- or sorbitol-induced Elk-1 reporter activation. CV-1 cells were transfected with Gal4–LUC or Gal4–Elk-1 in the presence (hatched bars) or absence (open bars) of KSR. Cells were treated with EGF (50 ng/ml), TNF-α (10 ng/ml) or sorbitol (600 mM, see Materials and methods). Error bars indicate SD from experiments performed in triplicate. Shown is a representative example of at least three independent experiments. (**D**) Western blot for expression of KSR. The expression of transfected KSR was detected by anti-HA antibody. The samples are identical to those in panel C. (**E**) KSR inhibits EGF- and PMA-induced SAP1a and SAP2 activity. CV-1 cells were transfected with Gal4–Elk-1 (lanes 1–6), Gal4–SAP1a (lanes 7–12) or Gal4–SAP2 (lanes 13–18) together with Gal4–LUC and KSR as indicated. Open and hatched bars denote co-transfection of vector or KSR, respectively. Serum-starved cells were stimulated for 8 h with EGF (50 ng/ml) or PMA (100 nM) and luciferase activity was determined and normalized as in Figure 2A. Error bars indicate SD from experiments performed in duplicate. Shown is a representative example of at least three independent experiments.

ERK-induced Elk-1 activation or if KSR inhibits Elk activation in general, we examined the effect of KSR on other pathways that induce Elk-1 activation. TNF-α is known to activate the JNK pathway which can also result in Elk-1 phosphorylation and activation (Cavigelli *et al.*, 1995). KSR blocked TNF-α-stimulated Elk-1 activation (Figure 3C and D). Similarly, high osmolarity stimulates p38 activity and results in Elk-1 activation (Cavigelli *et al.*, 1995; Whitmarsh *et al.*, 1997). KSR also inhibits sorbitol-induced Elk-1 activation (Figure 3C), suggesting that the inhibitory activity of KSR is not dependent on ERK activation, but rather is specific to Elk-1. Therefore, KSR function is not limited to ERK-induced Elk-1 activation.

Inhibition of SAP1a and SAP2 by KSR

The TCF family of transcription factors consists of Elk-1, SAP1a and SAP2, all of which appear to be physiological substrates of MAP kinase. To test the effect of KSR on the transactivation activity of SAP1a and SAP2 we constructed Gal4 fusion proteins consisting of the yeast Gal4 DNA-binding domain fused to the C-terminal activation domain of SAP1a and SAP2. When these expression vectors were introduced into CV-1 cells and subsequently stimulated with EGF or PMA, we observed a large increase in normalized luciferase activity (Figure 3E). Co-expression of KSR effectively blocked basal, EGF- and PMA-stimulated reporter activity for all members of the TCF family (Figure 3E). These results demonstrate that KSR inhibits the activity of all members of the TCF family.

An intact kinase domain of KSR is required for the inhibition of Elk-1

All protein kinases contain a conserved, essential lysine residue in subdomain II (Hanks and Quinn, 1991). Interestingly, the murine KSR contains an arginine (Arg589) in place of this conserved lysine. To determine if the kinase activity of KSR was required for its ability to block Elk-1 reporter, Arg589 was mutated to either a lysine or a methionine residue to generate KSR-RK and KSR-RM, respectively. We tested the effects of KSR-RM and KSR-RK on Gal4–Elk-1 reporter activity. Co-transfection of

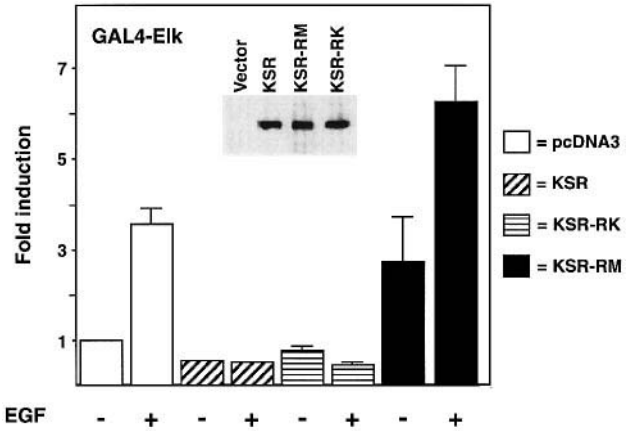

Fig. 4. KSR-RM enhances Elk-1 activity. CV-1 cells were transfected with Gal4–Elk-1 and Gal4–LUC, as above, together with the indicated KSR mutant. Serum-starved cells were stimulated with EGF for 8 h prior to harvesting and determination of luciferase activity. Luciferase activity was normalized for transfection efficiency as above. Error bars indicate SD from experiments performed in triplicate. A Western blot of KSR expression is shown as an insert in the figure. Shown is a representative example of at least three independent experiments.

wild-type KSR or KSR-RK severely inhibited basal and EGF-induced Gal4–Elk-1 reporter activity (Figure 4). In contrast, KSR-RM expression greatly enhanced both basal and EGF-stimulated reporter activity (Figure 4). Similarly, the KSR-RM mutant did not inhibit EGF-induced Elk-1 phosphorylation (Figure 5A, lane 9 in panel pElk-1). Since the KSR-RK mutant behaves identically to wild-type KSR to block Elk-1 phosphorylation and activity, and KSR-RM elevates Elk-1 activity, we conclude that the arginine to lysine substitution is still functional, but substitution to a methionine results in a loss of function, almost certainly due to loss of kinase activity. It is interesting to point out that both *Drosophila* and *C.elegans* KSR contains a lysine at this position. Our results suggest that the integrity of the kinase domain is essential for KSR's ability to block Elk-1 phosphorylation.

KSR blocks Elk-1 phosphorylation without affecting ERK phosphorylation

Phosphorylation of Ser383 has been shown to be critical for the transcription activity of Elk-1 (Gille *et al.*, 1992, 1995; Janknecht *et al.*, 1993; Marias *et al.*, 1993; Kortenjann *et al.*, 1994). KSR could inhibit Elk-1 activation by either directly blocking Elk-1 phosphorylation or indirectly repressing Elk-1 activity. To examine the effect of KSR on Elk-1 phosphorylation directly, an EGF-stimulated time course of Elk-1 phosphorylation was performed using a phosphorylation state-specific Elk-1 antibody, which recognizes only the Ser383 phosphorylated form of Elk-1. Data in Figure 5A show that Elk-1 phosphorylation was acutely stimulated by EGF with maximum phosphorylation occurring at 5 min. Co-transfection of KSR completely blocked EGF-stimulated Elk-1 phosphorylation (Figure 5B, panel pElk-1). Western blots with anti-Elk-1 showed that similar amounts of Elk-1 were expressed in all samples (Figure 5B, panel Elk-1). The kinase-deficient KSR-RM mutant was unable to block Elk-1 phosphorylation (Figure 5B, lane 9). In parallel, the same cell lysates were probed with antibody specifically recognizing phosphorylated ERK. KSR did not affect EGF-stimulated HA-ERK1 phosphorylation (Figure 5B, panel pERK) which was co-transfected with Elk-1 and KSR. The inhibition of Elk-1 phosphorylation by KSR was supported by *in vivo* ^{32}P-labeling and immunoprecipitating HA-Elk-1 from cells expressing KSR or vector alone. We observed a significant decrease in ^{32}P-incorporation in Elk-1 in cells co-expressing KSR, although immunoblotting revealed identical amounts of Elk-1 in each immune-complex (Figure 5C).

Co-expression of RasV12 or an active MEK1 mutant also resulted in elevated Elk-1 phosphorylation (Figure 5C). Therefore, we next examined the effect of KSR on Ras and MEK-induced Elk-1 phosphorylation. KSR completely inhibited active Ras or MEK1-induced Elk-1 phosphorylation (Figure 5C). Dominant-negative MEK1 expression blocked EGF-induced Elk-1 phosphorylation (Figure 5C). Similarly, the MEK inhibitor PD98059 also inhibited EGF-stimulated Elk-1 phosphorylation (data not shown), suggesting that MEK–ERK kinases are required for EGF-induced phosphorylation of Elk-1.

It is worth noting that expression kinase-deficient KSR, KSR-RM, did not inhibit Elk-1 phosphorylation (Figure 5A, lane 9, and 5D). In fact, co-transfection with KSR-RM resulted in an elevation of Elk-1 phosphorylation, suggesting that KSR-RM functions as a dominant interfering mutant. The effect of KSR-RM was not due to activation of ERK because KSR-RM did not increase either the basal or the stimulated ERK activity (Figure 1), and also did not increase ERK phosphorylation (Figure 5A, lane 9 in panel pERK). The observation that KSR-RM expression leads to an increase of Elk-1 phosphorylation is completely consistent with the ability of wild-type KSR to block Elk-1 phosphorylation. We examined the relationship between KSR and Ras further using the KSR-RM mutant. Expression of the RasN17 dominant-negative mutant (deVries-Smits *et al.*, 1992; Wood *et al.*, 1992) blocked EGF-induced Elk-1 phosphorylation (Figure 5D). Co-transfection with KSR-RM partially reversed the inhibitory effect of RasN17 (Figure 5D), supporting the hypothesis that KSR-RM functions downstream of Ras. These results lend further support to our above observations that a consequence of KSR expression is loss of Elk-1 activity via a dramatic decrease in Elk-1 phosphorylation.

KSR inhibits phosphorylation of cytoplasmic localized lacZ–ElkC

Based on current models of MAP kinase signaling, we reasoned that there are three known mechanisms that would explain our novel findings concerning KSR: (i) KSR expression may lead to a modification of ERK which renders it unable to phosphorylate Elk-1; (ii) KSR expression may lead to a modification of Elk-1 that renders it unsuitable as an ERK substrate and (iii) KSR may affect the localization of ERK and/or Elk-1.

We first tested whether KSR co-transfection alters the ability of ERK to phosphorylate Elk-1. ERK1 was immunoprecipitated from KSR-expressing cells and used to phosphorylate recombinant glutathione *S*-transferase (GST)–Elk-1. MBP was included as an internal control. Our results showed that ERK1 in KSR co-transfected cells was fully activated by EGF, and that the activated ERK1 can effectively phosphorylate GST–Elk-1 *in vitro* (Figure

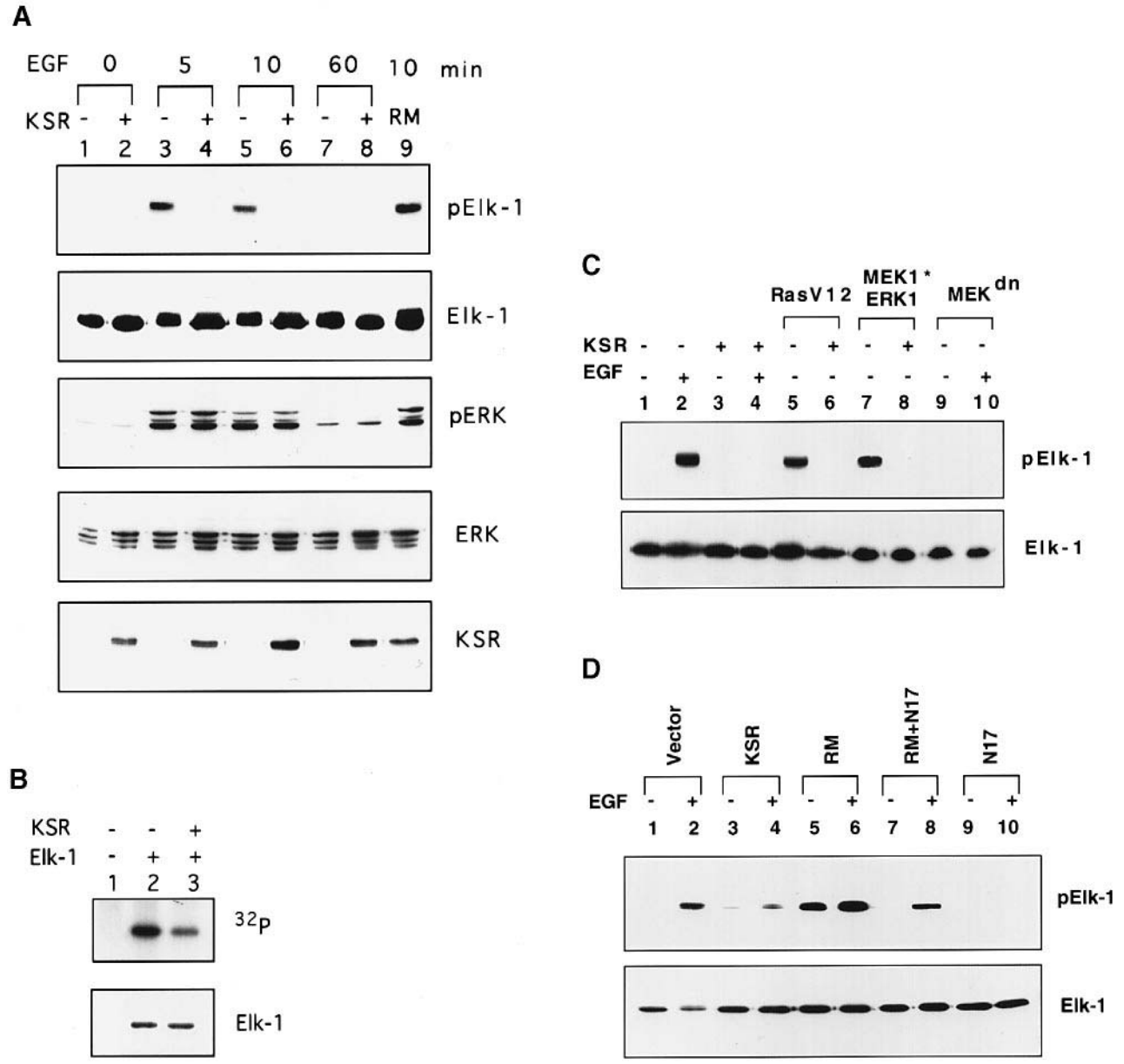

Fig. 5. KSR selectively blocks Elk-1 but not ERK phosphorylation. (**A**) The kinase activity of KSR is required to block Elk-1 phosphorylation. COS1 cells were transfected with 0.5 μg of pcDNA3-Elk-1, 0.5 μg HA-ERK1 and 1.0 μg of either pcDNA3 (lanes 1, 3, 5 and 7), pcDNA3-HA-KSR (lanes 2, 4, 6 and 8) or KSR-RM (lane 9). Cells were treated with EGF for the indicated times. Cell lysates were subjected to immunoblot analysis with anti-pElk-1, anti-Elk-1, anti-pERK, anti-ERK or anti-HA (for HA-KSR) as indicated on the right side of each panel. Anti-Elk-1 and anti-pElk-1 antibodies specifically recognize the unphosphorylated and phosphorylated Elk-1, respectively. Anti-pERK antibody only recognizes the phosphorylated ERK while anti-ERK antibody recognizes both the phosphorylated and unphosphorylated ERK. The three bands detected by the anti-ERK antibody are due to endogenous ERK1 and ERK2 (the lower two bands) and the transfected HA-ERK1 (upper band). (**B**) KSR blocks Elk-1 phosphorylation. COS1 cells were transfected with HA-Elk-1 with or without KSR, labeled with [^{32}P]-PO_4 (0.5 mCi/ml), for 4 h and stimulated with EGF for 5 min. HA-Elk-1 was immunoprecipitated using the anti-HA antibody. Immunoprecipitated HA-Elk-1 was subjected to autoradiography (upper panel) or immunoblot with anti-Elk-1 (lower panel). (**C**) KSR blocks Ras and MEK1 induced Elk-1 phosphorylation. COS1 cells were transfected with 0.5 μg pcDNA3-Elk-1 together with 1.0 μg of pcDNA3-HA-KSR, RasV12, MEK1* plus ERK1, or MEKdn as indicated. Cell lysates were probed with either anti-pElk-1 (upper panel) or anti-Elk-1 (lower panel). (**D**) KSR-RM functions as a dominant interfering mutant and enhances Elk-1 phosphorylation. Elk-1 was co-transfected with 1.0 μg of either vector (lanes 1 and 2), pcDNA3-HA-KSR (lanes 3 and 4), KSR-RM (lanes 5 and 6), RasN17 (lanes 9 and 10) or KSR-RM plus RasN17 (lanes 7 and 8). After serum starvation, transfected cells were left untreated or treated with EGF (5 min). Immunoblots were performed with either anti-pElk-1 (upper panel) or anti-Elk-1 (lower panel).

6A) as determined by both ^{32}P-incorporation and anti-phospho-Elk-1-specific antibody. The ratio of phosphorylation of Elk-1 over MBP was not decreased by co-transfection with KSR. These data exclude the possibility that KSR alters the substrate specificity of ERK1.

To test whether KSR decreased the efficacy of Elk-1 to serve as an ERK substrate, an *in vitro* phosphorylation assay of Elk-1 immunoprecipitated from KSR expressing cells was performed. HA-Elk-1 was co-transfected with KSR or the kinase-deficient RM mutant. HA-tagged Elk-1 was immunoprecipitated from serum-starved cells and subjected to *in vitro* phosphorylation by recombinant active ERK1. Immunoprecipitated HA-Elk-1 was readily phosphorylated by recombinant ERK1 *in vitro* regardless

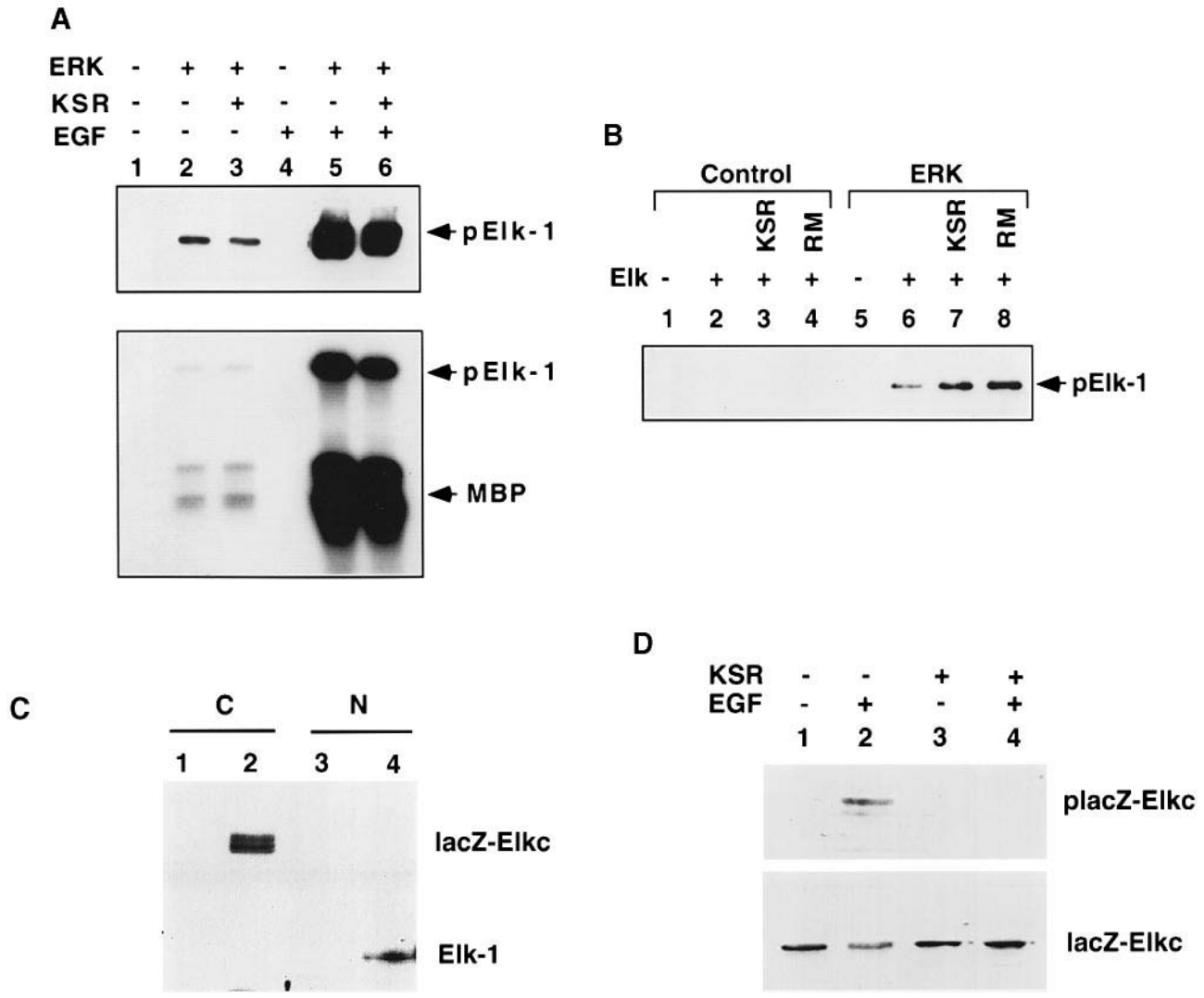

Fig. 6. KSR blocks cytoplasmic localized lacZ–ElkC phosphorylation. (**A**) KSR does not affect the ability of ERK to phosphorylate Elk-1 *in vitro*. HA-ERK1 (0.5 μg) was co-transfected with 1 μg of vector or KSR. The transfected cells were serum-starved and stimulated with EGF for 5 min as indicated. HA-ERK1 was immunoprecipitated and used to phosphorylate 2 μg of recombinant GST–Elk-1 with cold ATP. Phosphorylation of GST–Elk-1 was detected by the anti-pElk-1 antibody (upper panel). The immunoprecipitated HA-ERK1 was also used to phosphorylate GST–Elk-1 (2 μg) together with myelin basic protein (MBP, 20 μg) in the presence of [^{32}P]γ-ATP. Phosphorylation of GST–Elk-1 and MBP was detected by autoradiography (lower panel). (**B**) KSR does not affect the ability of Elk-1 to serve as an ERK substrate. HA-Elk-1 (0.5 μg) was co-transfected with 1μg KSR or KSR-RM. HA-Elk-1 was immunoprecipitated and subjected to *in vitro* phosphorylation by buffer control (lanes 1–4) or 10 ng ERK1 (lanes 5–8). The phosphorylation of HA-Elk-1 was detected by anti-pElk. The higher level of pElk-1 in lanes 7 and 8 was due to a comparably higher level of Elk-1 in the immunoprecipitation (data not shown). (**C**) lacZ–ElkC fusion is cytoplasmic. pcDNA3-lacZ–ElkC (0.5 μg) was co-transfected with pcDNA3-Elk-1 and KSR into COS1 cells. Cells were fractionated into cytoplasmic (C) and nuclear (N) fractions and probed with anti-Elk-1 antibody. Lanes 1 and 3 are transfected with empty vector while lanes 2 and 4 are transfected with pcDNA3-lacZ–ElkC together with pcDNA3-Elk-1. lacZ–ElkC is cytoplasmic (lane 2) while Elk-1 is nuclear (lane 4). (**D**) KSR blocks the phosphorylation of cytoplasmic lacZ–ElkC. pcDNA3-lacZ–ElkC transfected COS1 cells were stimulated with EGF for 5 min as indicated. Western blot was performed with anti-pElk-1 (top panel, detection of phosphorylation) or anti-Elk-1 (bottom panel, detection of expression). EGF induced a rapid lacZ–ElkC phosphorylation (lane 2) which was inhibited by KSR (lane 4).

of whether KSR was co-expressed (Figure 6B). Thus, KSR expression does not affect the ability of Elk-1 to be phosphorylated by recombinant ERK1, nor does it block the recognition of the anti-pElk-1 epitope.

Next we tested the third hypothesis: whether KSR alters the localization of ERK and/or Elk-1. Immunofluorescence and subcellular fractionation of transfected HA-ERK1 or HA-Elk-1 in KSR-expressing cells indicated that the subcellular localization of neither HA-ERK1 nor HA-Elk-1 was altered (data not shown). We reasoned that if KSR affects nuclear translocation of ERK, the inhibitory effect of KSR on Elk-1 phosphorylation should be confined to the cell nucleus. To test this possibility, the C-terminal ERK-responsive domain of Elk-1 was fused to lacZ in a mammalian expression vector. When COS1 cells were co-transfected with lacZ–ElkC and wild-type Elk-1, lacZ–ElkC fusion was localized to the cytoplasm (Figure 6C). In contrast, wild-type Elk-1 was nuclear as determined by cell fractionation and immunoblotting (Figure 6C). Phosphorylation of Elk-1 serine 383 in lacZ–ElkC was acutely stimulated by EGF identical to the wild-type Elk-1 (Figure 6D), suggesting that the lacZ–ElkC is phosphorylated by ERK in a stimulation-dependent manner. Co-expression of KSR effectively inhibited the

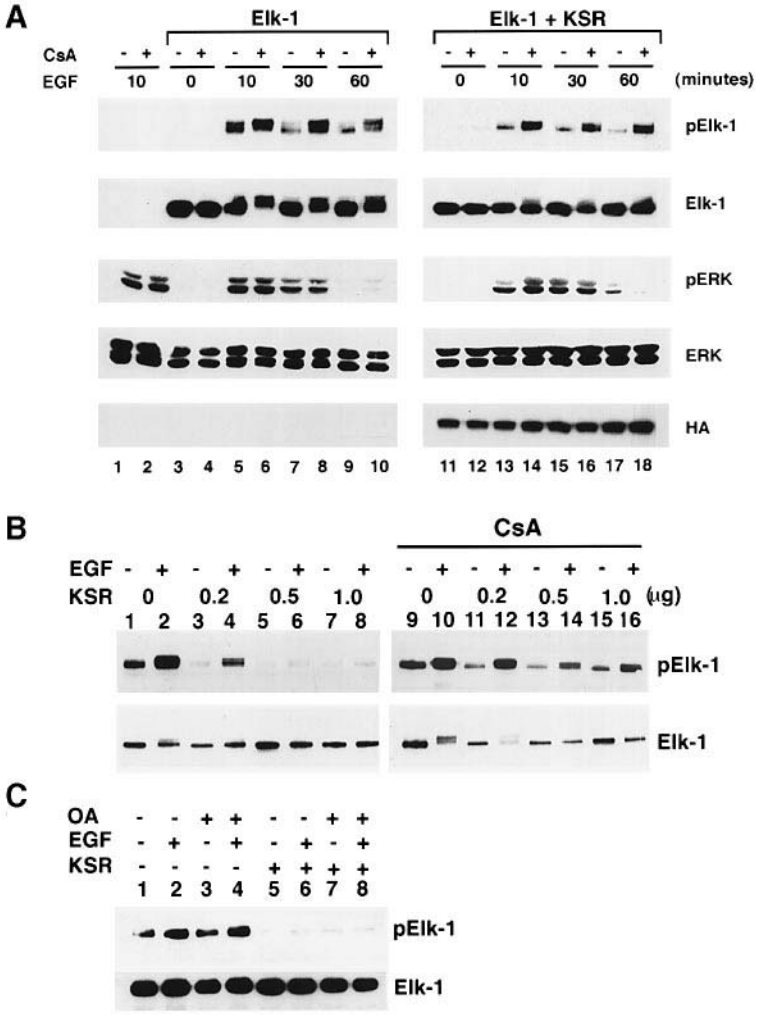

Fig. 7. Cyclosporin A blocks the effect of KSR. (**A**) Cyclosporin A blocks the inhibitory effect of KSR. COS1 cells were transfected with pcDNA3-Elk-1 with or without pcDNA3-HA-KSR (1.0 μg) as indicated. Cells were starved with 0.1% serum for 24 h and stimulated with EGF for the indicated times. Cyclosporin A (5 μM) was added 30 min prior to the addition of EGF (even numbered lanes). Samples were blotted with the indicated antibodies. Transfected KSR was detected with anti-HA antibody. (**B**) Cyclosporin A reverses the inhibitory effect of KSR on Elk-1 phosphorylation. pcDNA3-Elk-1 (0.5 μg) was co-transfected with the indicated amounts of pcDNA3-HA-KSR. After serum starvation, COS1 cells were left untreated (lanes 1–8) or treated (lanes 9–16) with CsA (5 μM) for 30 min. EGF stimulation was 10 min. Immunoblots were performed with either anti-pElk-1 (top panel) or anti-Elk-1 (bottom panel). (**C**) Okadaic acid has no detectable effect on KSR function. COS1 cells were transfected with pcDNA3-Elk-1 together with KSR as indicated. The cells were starved as described in Figure 6A and pre-incubated with 1 μM Okadaic acid for 30 min (lanes 3, 4, 7 and 8), followed by stimulation with EGF for 10 min. Cell lysates were probed with either anti-pElk-1 (top panel) or anti-Elk-1 (bottom panel).

phosphorylation of cytosolic lacZ–ElkC. These results suggest that the inhibitory effect of KSR is not confined to the nucleus, inconsistent with the possibility that KSR blocks the nuclear translocation of ERK.

Cyclosporin A blocks the effect of KSR

We examined the effects of phosphatase inhibitors on the ability of KSR to inhibit Elk-1 serine 383 phosphorylation. Okadaic acid, an inhibitor of phosphoprotein phosphatases 1 and 2A (PP1 and PP2A), was used in conjunction with cyclosporin A (CsA), an inhibitor specific to calcineurin, also known as PP2B, in order to begin to understand the mechanism by which KSR functions. COS1 cells were transfected with Elk-1 and KSR and stimulated with EGF in the presence or absence of CsA. Addition of CsA significantly reduced the inhibitory effect of KSR on Elk-1 phosphorylation (Figure 7A), particularly when lower amounts of KSR expression vector were used (Figure 7B). Identical experiments using okadaic acid showed no effect on Elk-1 phosphorylation (Figure 7C). Importantly, the effect of CsA on Elk-1 phosphorylation was not due to ERK activation, as ERK phosphorylation is not affected by either KSR expression or CsA pre-treatment (Figure 7A). This observation suggests a possible role for calcineurin in mediating Elk-1 inactivation by KSR.

Discussion

KSR as a novel regulator of MAP kinase signaling pathways

The Ras–MAP kinase pathway is one of the most widely utilized signaling cascades in eukaryotic cells. Its function has been implicated in many diverse signal transduction events ranging from cell growth, differentiation and survival to oncogenic transformation (Robinson and Cobb, 1997). Previously, components identified in this pathway

have been shown to modulate activities of other enzymes in the pathway directly. Phosphorylation of cellular targets by ERK obviously plays a critical role for signaling in this pathway. Phosphorylation and activation of the TCF family transcription factors by MAP kinases have been shown to mediate transcriptional activation of cellular genes that are targets of the Ras–MAP kinase pathway. Here we demonstrate that a hitherto undescribed mechanism exists by which KSR is able to modulate the phosphorylation status of Elk-1 without directly altering the activity of ERK1. The observation that Elk-1 is not phosphorylated in the presence of KSR even though ERK is fully phosphorylated and activated suggests that KSR can uncouple Elk-1 phosphorylation from ERK activation. Phosphorylation of Elk-1 was determined by anti-phospho-Elk-1-specific antibody, ^{32}P-labeling and functional analysis of Elk-1-dependent transcription. Similarly, ERK activation was determined by both phosphorylation and kinase activity. The effect of KSR is specific to Elk-1 because KSR does not block RacV12-induced Gal4–Jun activation. Recently it was reported that KSR is activated by ceramide and can directly phosphorylate and activate Raf (Zhang *et al.*, 1997). We were unable to detect Raf-1 activiation by KSR even though similar experimental conditions were used. We have no explanation for these discrepancies.

The observations described in this report are likely to be physiologically relevant, as opposed to being simply due to an artifact of transient transfection/overexpression, for the following reasons. First, KSR can block the expression of the c-fos promoter induced by both oncogenic Ras and growth factors, which uses endogenous TCF transcription factors. Secondly, the KSR-RM mutant does not inhibit Elk-1 phosphorylation, rather it serves to enhance the phosphorylation of Elk-1. These results suggest that KSR-RM functions as a dominant interfering mutant and more importantly, are completely consistent with the observations that a function of wild-type KSR is to block the activation of Elk-1. Thirdly, the effect of KSR is specific to the TCF family. Fourthly, KSR also inhibits TNFα- and sorbitol-induced Elk-1 activation. Finally, the inhibitory effect of KSR was observed in several cell lines, including COS1, CV1 and NIH-3T3. Our studies established that KSR acts as a negative regulator of Elk-1 phosphorylation in response to growth factor and Ras signaling.

Our data support the model that KSR functions downstream of, or parallel to, the Ras–MAP kinase pathway and uncouples Elk-1 phosphorylation from ERK activation (Figure 8). This can be achieved by either preventing Elk-1 phosphorylation or promoting dephosphorylation. Our results are consistent with the latter possibility. KSR affected neither the activation of ERK1 nor the ability of ERK1 to phosphorylate Elk-1. The subcellular localization of ERK1 is apparently also not affected by KSR. In addition, KSR does not decrease the efficacy of Elk-1 to be phosphorylated by recombinant ERK. Our results presented here argue against the model that KSR directly inhibits Elk-1 phosphorylation. However, our data are consistent with the hypothesis that KSR may promote the accumulation of dephosphorylated Elk-1 via the action of a phosphatase rather than inhibiting the activities of Elk-1 kinases directly. We have recently observed that

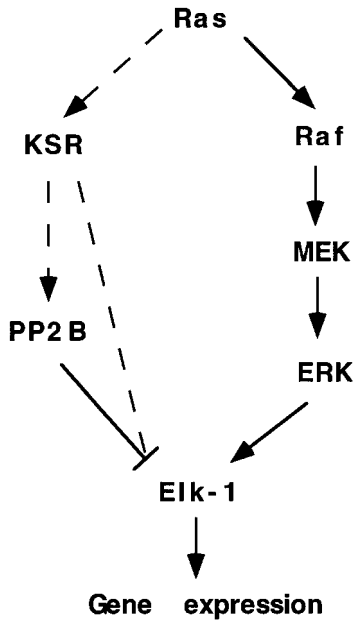

Fig. 8. A proposed model of KSR in regulation of Elk-1. (→) and (—|) denote positive and negative actions, respectively. Dashed lines indicate possible indirect effects. KSR inhibition of Elk-1 phosphorylation could be mediated by PP2B (phosphoprotein phosphatase 2B, also known as calcineurin) dependent or independent pathways.

calcineurin is the major Elk-1 phosphatase (Sugimoto *et al.*, 1997). Reversal of the inhibitory effects of KSR on Elk-1 by inhibition of calcineurin supports the proposed model and suggests a role for calcineurin in both KSR and Elk-1 function (Figure 8). The fact that KSR blocked the ability of different pathways (JNK and p38) to induce Elk-1 phosphorylation is also consistent with the notion that KSR promotes Elk-1 dephosphorylation. We propose that both inactivation of KSR and activation of ERK are required to achieve maximum activation/phosphorylation of Elk-1 during receptor tyrosine kinase and Ras signaling which induce the expression of c-fos. This report provides the first example showing that TCF activity can be regulated independently of MAP kinase activity *in vivo*.

KSR and differential signal output of the MAP kinase pathway

Genetic studies demonstrate that KSR plays a positive role in both Ras-induced vulval development of *C.elegans* and photoreceptor development of *Drosophila* (Kornfeld *et al.*, 1995; Sundaram and Han, 1995; Therrien *et al.*, 1995). Mutations of KSR suppress the phenotype of a constitutively active mutant of Ras in both processes. We report here that KSR has a negative role in growth factor and Ras-induced Elk-1 activation in mammalian cells. Interestingly, Elk-1 belongs to the Ets family of transcription factors which have been implicated in cell proliferation (reviewed in Macleod *et al.*, 1992). Elk-1 may play a role in Ras-induced cell proliferation rather than differentiation. Lin-1 (Beitel *et al.*, 1995) and Yan (Rebay and Rubin, 1995) are Ets-containing proteins involved in *C.elegans* vulval induction and *Drosophila* photoreceptor differentiation, respectively. Both Lin-1 and Yan are negative regulators in Ras-mediated differentiation. Therefore, inhibition of Ets-containing proteins might serve to

promote cell differentiation. Our data provide a possible biochemical mechanism for KSR function in Ras–MAP kinase signal transduction.

It is well established and perplexing that Ras can promote both cell proliferation and differentiation in different cell types in response to various extracellular signals. KSR could play a critical role in suppressing the proliferative effect of Ras, thereby promoting Ras-induced differentiation. MAP kinases are activated by a myriad of extra-cellular stimuli. However, the mechanism for generating different physiological responses by activation of a common set of signaling molecules is far from understood. The function of KSR may be to specifically affect a subset of MAP kinase substrates without affecting the phosphorylation of others, thus producing different cellular responses from a common MAP kinase cascade.

Materials and methods

Cell culture and transfection

COS1, CV-1 and NIH-3T3 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS). Transfection of COS1, CV-1 and NIH-3T3 cells was done by DEAE-Dextran (Braiser, 1994) and LipofectAMINE (Gibco-BRL) methods, respectively. 24 h after transfection, cells were starved for an additional 24 h by incubating with DMEM containing 0.1% FBS prior to stimulation.

Plasmids

All DNA manipulations were performed essentially as described (Sambrook *et al.*, 1989). To construct epitope-tagged expression plasmids, we first inserted a DNA fragment encoding a start methionine followed by three tandem repeats of the hemagglutinin antigen (HA) epitope (YPYDVPDYA) between the *Hin*dIII and *Not*I sites of pcDNA3 (Invitrogen) to produce the pcDNA3-HA vector. To express HA-epitope tagged KSR (HA-KSR), the entire coding region of KSR was amplified by the polymerase chain reaction (PCR) using murine KSR cDNA as a template (a generous gift of Dr Gerald Rubin). The PCR product was subcloned into pcDNA3-HA at the unique *Xba*I–*Eco*RI sites. KSR was also subcloned into pcDNA3 without an epitope tag. The *Xba*I–*Eco*RI fragment of KSR coding sequence was also subcloned into pALTER-1 (Promega). Point mutations of KSR were made using the Altered Sites II *in vitro* mutagenesis system (Promega). Mutations were confirmed by DNA sequencing. Mutant KSRs was subcloned into pcDNA3-HA similar to the wild-type KSR.

To express HA-tagged MEK1 (HA-MEK1), the *Bam*HI fragment of pGEX-2T-MEK1 (Zheng *et al.*, 1993) was subcloned into pcDNA3-HA at the *Bam*HI site. The HA-MEK1 plasmid will express MEK1 with two HA repeats at its N-terminus. To construct an Elk-1 expression vector, the human Elk-1 cDNA was subcloned into pcDNA3 or pcDNA3-HA. A constitutively active human MEK1d* was made by deletion of amino acid residues 32–51 and replacement of Ser218 and 222 by glutamic acid residues (Mansour *et al.*, 1994). The MEK1* contains the Ser218 and 222 to glutamic acid only. The dominant-negative form of MEK1dn was made by changing the Ser218 and 222 to alanines. These MEK1 mutants were subcloned into the pCMV5 vector (Andersson *et al.*, 1989). To construct a lacZ–ElkC expression vector, a cDNA fragment which corresponds to the C-terminus of Elk-1 (amino acids residues 305–425) was subcloned into the lacZ expression vector pcDNA3.1(–)/Myc-His/lacZ (Invitrogen).

The activation domains of SAP1a (amino acid residues 323–430) and SAP2 (amino acid residues 299–407), containing all conserved MAP kinase phosphorylation sites, were amplified by RT–PCR from human placenta mRNA and subcloned into a mammalian expression vector in-frame with a Gal4 DNA binding domain.

Kinase assays

COS1 cells were transfected with HA-c-Raf, HA-ERK1 or HA-MEK1 in the presence or absence of KSR. The transfected cells were serum starved for 24 h followed by stimulation with 10% FBS or EGF (50 ng/ml). Transfected cells were lysed in buffer (10 mM Tris–Cl pH 7.5, 2 mM EDTA, 150 mM NaCl, 1% NP-40, 50 mM NaF, 1 mM sodium vanadate, 1 mM PMSF and 10 μg/ml aprotinin). HA tagged kinases were incubated with 1 μg of 12CA5 antibody (anti-HA) for 2 h at 4°C followed by addition of protein A agarose (Pierce) for 1 h. Immunoprecipitated kinases were washed three times with lysis buffer followed by one wash with 25 mM HEPES pH 7.5, 1 mM DTT and 0.5 mM EDTA. Kinase activities for Raf, MEK and ERK were measured as described previously (Zheng *et al.*, 1994). GST–MEK1 and GST–ERK1 were expressed in *Escherichia coli* and purified as described previously (Zheng and Guan, 1993). GST–Elk-1 (containing the C-terminal amino acid residues 305–425) was expressed in *E.coli* and purified by glutathione affinity chromatography.

Reporter analysis

Gal4–Elk-1, which contains the C-terminal transactivation domain fused to the Gal4 DNA binding domain, was co-transfected with Gal4–LUC, which has the luciferase expression under the control of four tandem repeats of the Gal4 DNA binding sequence. A pCMV-lacZ plasmid was co-transfected as an internal control for transfection efficiency and sample handling. 24 h after transfection, cells were starved in 0.1% FBS for 24 h followed by stimulation with EGF (50 ng/ml) or TNF-α (10 ng/ml) for 6–8 h. Cells were lysed in lysis buffer (Braiser, 1994). Luciferase activity were measured as described (Braiser, 1994). Co-transfected β-galactosidase activity was also determined and used to normalize the luciferase activity. When co-transfections were performed with Ras or MEK1, the transfected cells were also starved in 0.1% FBS medium for 24 h. However, cells were directly harvested and assayed for luciferase and β-galactosidase activities without EGF or serum stimulation. For sorbitol stimulation, transfected cells were starved in 0.1% FBS medium for 24 h and cells were incubated with 600 mM sorbitol for 1 h. The stimulated cells were cultured in 0.1% FBS medium without sorbitol for an additional 7 h before harvesting and assaying for reporter activity.

Cell fractionation

To separate the nuclear and cytoplasmic fractions, transfected COS1 cells (100 mm plate) were washed twice with phosphate buffered saline and scraped into 1 ml of hypotonic lysis buffer (10 mM β-glycerolphosphate, 2 mM $MgCl_2$, 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 1 mM sodium vanadate, 40 μg/ml PMSF, 10 μg/ml leupeptin, 10 μg/ml pepstatin). Cell fractionation was performed as described previously (Chen *et al.*, 1992; Zheng and Guan, 1994). Both the nuclear and cytoplasmic fractions were resuspended in equal volume (1 ml) of the lysis buffer and subjected to SDS–PAGE and immunoblot analysis.

Immunoblot analysis

COS1 cells were directly lysed in SDS sample buffer, subjected to SDS–PAGE and transferred to a PVDF membrane (Millipore). Typically, a 35 mm plate was lysed in 150 μl SDS sample buffer. After electrophoresis and transfer, the membranes were probed as indicated in the figure legends with various antibodies. Immunoreactive bands were detected by the ECL detection kit (Amersham). Anti-ERK1 antiserum was prepared against recombinant proteins (Zheng and Guan, 1994). Anti-HA antibody was prepared using the 12CA5 hybridoma cell line. Anti-Elk-1 and pElk-1 were purchased from New England Biolabs. Anti-pERK antibody was purchased from Promega.

Treatment with phosphatase inhibitors, in vivo labeling

To determine the effect of phosphatase inhibitors on KSR function, Elk-1 transfected cells were treated with 1 μM of okadaic acid (Calbiochem) or 5 μM of cyclosporin A (Sigma). After 30 min treatment with phosphatase inhibitors, cells were stimulated with EGF for the indicated times. Cells were harvested and subjected to immunoblots with various antibodies as described above.

For *in vivo* labeling, COS1 cells were transfected with HA-Elk-1 with or without KSR. 24 h after transfection, cells were starved in 0.1% FBS medium for 24 h. Cells were incubated further in phosphate free medium with 0.1% dialyzed serum (Gibco-BRL) in the presence of 0.5 mCi/ml ^{32}P for 4 h. After EGF stimulation for 5 min, cells were harvested. The labeled HA-Elk-1 was immunoprecipitated from cell lysates by anti-HA antibody (as described in the kinase assays) and subjected to autoradiography or Western blot with anti-Elk-1 antibody.

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