Kinase Suppressor of Ras Inhibits the Activation of Extracellular Ligand-regulated (ERK) Mitogen-activated Protein (MAP) Kinase by Growth Factors, Activated Ras, and Ras Effectors*

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Kinase suppressor of Ras (KSR) is a loss-of-function allele that suppresses the rough eye phenotype of activated Ras in Drosophila and the multivulval phenotype of activated Ras in Caenorhabditis elegans. Genetic and biochemical studies suggest that KSR is a positive regulator of Raf signaling that functions between Ras and Raf or in a pathway parallel to Raf. We examined the effect of mammalian KSR expression on the activation of extracellular ligand-regulated (ERK) mitogen-activated protein (MAP) kinase in fibroblasts. Ectopic expression of KSR inhibited the activation of ERK MAP kinase by insulin, phorbol ester, or activated alleles of Ras, Raf, and mitogen and extracellular-regulated kinase. Expression of deletion mutants of KSR demonstrated that the KSR kinase domain was necessary and sufficient for the inhibitory effect of KSR on ERK MAP kinase activity. KSR inhibited cell transformation by activated RasVal12 but had no effect on the ability of RasVal12 to induce membrane ruffling. These data indicate that KSR is a potent modulator of a signaling pathway essential to normal and oncogenic cell growth and development.

Ras proteins are integrators of diverse extracellular signals that regulate cell growth and development (1). In turn, Ras proteins regulate multiple downstream signal transduction pathways through direct protein-protein interactions (2–4). One pathway regulated by Ras involves the sequential activation of the cytoplasmic kinases Raf, MEK1,† and ERK (5, 6). Genetic screens for additional downstream effectors of Ras in Drosophila and Caenorhabditis elegans lead to the identification of the loss-of-function allele kinase suppressor of Ras (7–9). Epistasis experiments indicated that KSR was a positive regulator of Ras signaling acting in series between Ras and Raf or in parallel to Raf. Results from these genetic studies were further supported by biochemical studies examining the function of KSR (10). Murine KSR cooperated with activated RasVal12 (10) or with 14-3-3 proteins (11) to enhance the maturation of Xenopus oocytes. KSR expression enhanced the biological activity of RasVal12 by accelerating the kinetics of Raf activation (11), MEK1, and ERK MAP kinase activation (10), and this effect could be inhibited by introduction of dominant-negative Raf (11). Surprisingly, the cooperative activity of KSR was localized, not to its putative kinase domain, but to a noncatalytic amino-terminal region including a conserved, cysteine-rich area termed CA3 (12).

In the experiments reported here we have examined the ability of KSR to affect the activation of ERK MAP kinase by growth factors, activated Ras, and activated effectors of Ras. We observed that the ectopic expression of KSR resulted in the inhibition of ERK MAP kinase activation. Furthermore, this inhibitory effect of KSR on ERK MAP kinase activity resulted in the suppression of cell transformation by activated Ras and could be mimicked by expression of the KSR kinase domain alone. These data suggest that distinct structural domains within KSR may differentially modulate signal transduction between Raf, MEK, and ERK MAP kinase.

EXPERIMENTAL PROCEDURES

Cell Culture—Cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and incubated at 37 °C. 293T cells were grown in an atmosphere of 5% CO2. REF-52 cells were grown in 6% CO2.

Construction of KSR Expression Plasmids—The FLAG epitope tag was added to the COOH terminus of KSR using a PCR strategy with pMA57 cDNA (KSR in pBS) as the template. The primers were 5'-CTCTGGACATCTAATAAGACTAG-3' and 5'-CGGGGACCTCACCTTGTACGTCATCGTCTCTGTTGATGCAATCGCCTCAGG-3'. The PCR product was digested with NsiI and KpnI and then ligated into pCMV5 after NsiI and KpnI digestion to give plasmid pCMV5/KSR. The pCMV5/KSR cDNA was cut with EcoRI and KpnI to produce pCMV5/KSR.

For the construction of the KSR expression plasmid, a priming restriction enzyme site was made to allow for the Ksr cDNA fragment to be ligated into the plasmid vector. The two primers were annealed together, cut with EcoRI and ApaI and then ligated into pCMV5/KSR cut with EcoRI and ApaI. EcoRI and ApaI digestion of pCMV5/KSR removed the portion of the cDNA encoding the NH2 terminus of KSR. pMA57 cDNA was used as a template to create KSR N539

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† The abbreviations used are: MEK, mitogen and extracellular-regulated kinase; ERK, extracellular ligand-regulated kinase; KSR, kinase suppressor of Ras; MAP, mitogen-activated protein; FMA, phorbol 12-myristate 13-acetate; Raf-CAAX, c-Raf-1 kinase targeted to the plasma membrane by a carboxyl-terminal lipid modification signal from Ha-ras; MEKEE, constitutively active MEK; PCR, polymerase chain reaction.
using a PCR strategy. The upstream primer was 5′-GCAGGAAATTC-CCTCGGCGTTCCTCGCAG-3′ and the downstream primer, which added the FLAG epitope and an XbaI restriction site to the 3′ end, was 5′-GCTCTAGATCACTTGTCATCGTCGTCCTTGTAGTCCCTCCAGG-GCGCGCGGCCGAC-3′. The PCR product was digested with EcoRI and XbaI to yield a 1722-base pair fragment. pCMV5 was also digested with EcoRI and XbaI and the PCR products ligated into these sites to produce pCMV5KSR N539. All recombinant DNA was sequenced to confirm the fidelity of each construct.

**ERK MAP Kinase Assay—** 293T cells were transfected with the indicated cDNAs using calcium phosphate (14). This procedure resulted in transfection efficiencies in 293T cells near 90%. Cells were deprived of serum for 48 h after transfection, then stimulated with 100 mM insulin or 1 μM PMA for 10 min and lysed. Clarified cell lysates were immunoprecipitated using ERK-1 and ERK-2 antibodies (Santa Cruz Biotechnology), and ERK MAP kinase assays were performed using myelin basic protein as a substrate (15). Reactions were stopped by the addition of sample buffer and heating to 110 °C for 3 min and then subjected to SDS-polyacrylamide gel electrophoresis on a 12% gel. Radiolabeled myelin basic protein was quantified using phosphor storage technology (Molecular Dynamics). Results are representative of at least four independent experiments in which similar extent of inhibition was obtained.

Focus Formation—NIH 3T3 cells were transfected by the calcium phosphate method (14) with either empty vector or pCGT RasVal-12 (500 ng) plus the indicated concentration of KSR for a total of 15.5 μg of total plasmid DNA/cm². Transfected cells were grown in media containing calf serum (5%) for 14 days, then fixed in 3.7% formaldehyde and stained with Giemsa. Foci of transformed cells appear as diffuse, darkly staining spots.

**RESULTS**

Epistasis experiments in *Drosophila* and *C. elegans* have suggested that KSR is a positive effector of Ras-mediated signals (7–9). The ability of KSR to affect ERK MAP kinase activity was assessed by transient expression of KSR in human embryonic kidney 293T cells. It is well established that insulin stimulates ERK MAP kinase (16) through activation of the Ras/Raf/MEK signaling pathway (17, 18). It is well established that insulin stimulates ERK MAP kinase (16) through activation of the Ras/Raf/MEK signaling pathway (17, 18). In agreement with these data, insulin induced an 8-fold activation of ERK MAP kinase in cells transfected with the human insulin receptor (Fig. 1A). In contrast, co-expressing KSR with the human insulin receptor resulted in the 6-fold inhibition of ERK MAP kinase activity. To determine whether the inhibitory effect of KSR on ERK MAP kinase activation is unique to the insulin signaling system, we tested the effect of KSR expression on ERK MAP kinase activation by the phorbol ester PMA. Phorbol esters bind to protein kinase C, which can phosphorylate and activate Raf independent of Ras to stimulate ERK MAP kinase (19). PMA treatment of 293T cells activated ERK MAP kinase activity 30-fold. However, expression of KSR also resulted in a 7-fold inhibition of PMA-stimulated ERK MAP kinase activity (Fig. 1B). These observations indicated that the mechanism of ERK MAP kinase inhibition by KSR was not due to its direct effect on growth factor receptors.

Intracellular signals are transmitted sequentially from Ras to Raf, and then to MEK, which phosphorylates and activates ERK MAP kinase (5). To determine the level in the signal transduction cascade at which KSR inhibits MAP kinase activation, 293T cells were transfected with Ras<sup>Ap-12</sup> (20), Raf-CAAX (21), or MEKEE (22). Expression of Ras<sup>Ap-12</sup>, Raf-CAAX, and MEKEE alone led to the activation of ERK MAP kinase, and this activation was abolished by co-transfection of KSR (Fig. 2A). This observation suggests that KSR inhibits the activation of ERK MAP kinase downstream of, or at the level of, MEK.

The action of KSR was further examined by co-injection of
FIG. 2. KSR inhibits ERK MAP kinase activation by activated forms of Ras, Raf, and MEK. A, 293T cells were transfected with control vector (pCMV5) and Ras^{Asp-12}, Raf-CAAX, or MEK alone or in combination with KSR. Expression of recombinant proteins was confirmed by probing Western blots of cell lysates with antibodies against the epitope tag added to Ras^{Asp-12} (\textit{α}myc), Raf-CAAX (\textit{α}Raf), MEK (\textit{α}MEK1), and the epitope tag added to KSR (\textit{α}FLAG). ERK MAP kinase activity was determined as described in the legend to Fig. 1. Results are representative of three independent experiments in which similar extent of inhibition was obtained. B, REF-52 cells were microinjected with control vector, Ras^{Val-12}, Raf-CAAX, or activated MEK alone or in combination with KSR. ERK MAP kinase activity was evaluated 3 h and 10 h post-injection by fixing and staining cells with antibodies specific for the phosphorylated ERK-1 and ERK-2. C, expression of KSR in microinjected REF-52 cells. REF-52 cells were fixed and stained with antibodies specific for the FLAG epitope 10 h post-injection.
KSR Inhibits ERK MAP Kinase Activation

FIG. 3. KSR does not inhibit RasVal-12-induced membrane ruffling. REF-52 cells were injected with pcGTrasVal-12 (5 ng/μl) and either KSR (100 ng/μl) or empty vector (100 ng/μl), 10 h after injection, cells were fixed and stained with Y13-259 anti-Ras antibodies. Results are representative of three independent experiments.

The ability of KSR to affect other Ras-mediated signaling pathways was also tested. Injection of fibroblast cells with activated Ras is known to induce membrane ruffling (16). Ras-mediated membrane ruffling and activation of ERK MAP kinase are mediated by distinct effectors (24). Co-injection of KSR had no effect on the ability of RasVal-12 to induce membrane ruffling (Fig. 3). This observation suggests that the inhibitory effect of intact KSR on ERK MAP kinase activation might be temporally regulated. Identical results were obtained when KSR was expressed without the FLAG epitope tag (data not shown).

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KSR consists of amino-terminal regions conserved between alleles from different species and a carboxyl-terminal kinase domain with greatest homology to the Raf kinase (7–9). Truncated forms of KSR, consisting of amino acids 1–539 (KSR N539) or the carboxyl-terminal kinase domain (amino acids 540–873, KSR C540) were tagged with the FLAG epitope and expressed in 293T cells or microinjected into REF-52 cells to determine each region’s contribution to the ability of KSR to suppress ERK MAP kinase activation. KSR N539 had no effect on the activation of ERK MAP kinase in 293T cells by phorbol esters (Fig. 4A), or in REF-52 cells by co-injection with RasVal-12 (Fig. 4B). In contrast, KSR C540 was a potent suppressor of ERK MAP kinase activation in both 293T and REF-52 cells (Fig. 4). These data demonstrate that expression of the KSR kinase domain alone is necessary and sufficient for the inhibition of ERK MAP kinase activation.

ERK MAP kinase activation contributes to the transforming potential of activated Ras. Mutated, constitutively activated members of this kinase cascade can induce transformation in certain cell types, whereas expression of kinase-deficient forms of these components interfere with the mitogenetic effects of Ras (6). To test the role of KSR in transformation, we co-transfected NIH 3T3 fibroblasts with expression plasmids for RasVal-12 and increasing concentrations of KSR. To visualize the foci, cells were fixed and stained with Giemsa 14 days after transfection. We found that focus formation of NIH 3T3 fibroblasts by RasVal-12 was inhibited in a dose-dependent manner by co-transfection of KSR (Fig. 5), which is consistent with its ability to suppress ERK MAP kinase activity.

DISCUSSION

In this report we have examined the ability of ectopic KSR to affect ERK MAP kinase activation by extracellular stimuli and upstream regulators. We find that murine KSR inhibits the activation of ERK MAP kinase by these factors. The ability of KSR to inhibit activation of ERK MAP kinase by activated Ras, Raf-CAAX, and MEK (Fig. 2) indicates that KSR exerts its inhibitory effect downstream or at the level of MEK. Consistent with its inhibitory effect on ERK MAP kinase activity, KSR inhibits the transforming potential of activated RasVal-12 when co-transfected into NIH 3T3 fibroblasts (Fig. 5). The inhibitory effects of KSR in this report contrast with observations that KSR facilitates Xenopus oocyte maturation and Balb/c 3T3 fibroblast transformation by RasVal-12 (10). The enhancing function of KSR has been attributed to the ability of the non-catalytic CA3 region in the amino-terminal portion of KSR to activate Raf (12). We were unable to detect a positive effect of the KSR amino-terminal domain on ERK MAP kinase activity when it was transiently expressed in 293T or REF-52 cells (Fig. 4). The delay between the time of KSR expression and the assay of ERK MAP kinase activity may conceal a positive effect of the KSR amino-terminal domain. Consistent with this possibility, co-injection of KSR did not inhibit the initial phase of ERK MAP kinase activation by RasVal-12, Raf-CAAX, or MEKEE (Fig. 2B). The delayed inhibitory effect of KSR raises the possibility that one role of KSR may be the feedback inhibition of activated ERK MAP kinase.

The inhibitory effect of KSR on ERK MAP kinase activation is consistent with the function of KSR in a pathway parallel to Raf (7) to deactivate ERK MAP kinase. Overexpression of KSR or the KSR kinase domain might constitutively activate the inhibitory program and down-regulate ERK MAP kinase signaling. The inhibitory effect of intact KSR on ERK MAP kinase activation was mimicked by transfection or microinjection of a truncated construct encoding the KSR kinase domain (Fig. 4). Our observations are consistent with the inhibition of RasVal-12-induced MEK and ERK activation in Xenopus oocytes and RasVal-12-induced Balb/c 3T3 transformation upon expression of the isolated KSR kinase domain in these cells (10). However, in that study full-length KSR did not have the same effect. Observations that an amino-terminal region of KSR facilitates Raf-activated pathways (10, 12) and that the carboxyl-terminal region of KSR inhibits ERK MAP kinase activation (Fig. 4) raise the possibility of cross-talk between these two domains of KSR. If the amino-terminal domain of KSR interacts with novel proteins to activate Raf (12), it is possible that the KSR kinase domain inhibits the stimulatory function of the amino-terminal domain by regulating the binding of those putative proteins. Regulation could occur through phosphorylation of the KSR amino-terminal region. The KSR kinase do-
main might also competitively inhibit the association of novel proteins with the KSR amino-terminal domain. Such an interaction could be independent of KSR kinase activity.

The inhibitory effect of KSR on ERK MAP kinase activation is also consistent with the predicted action of an ectopically expressed molecular scaffold. In *Saccharomyces cerevisiae*, Ste5p is a scaffold protein that binds yeast homologs of the mammalian ERK MAP kinase cascade (25). Although KSR bears no obvious structural homology to Ste5p, two-hybrid and biochemical analyses indicate that the KSR kinase domain can bind Raf (10) and MEK (26). A Ras-Raf-MEK complex has been shown to form in mammalian cells in advance of MEK1 activation (27). Scaffold proteins simultaneously associate with several effectors of a signaling pathway to create an ordered module that facilitates sequential activation of the associated enzymes (28). Overexpression of a KSR scaffold to a stoichiometry above the level of the available, endogenous catalytic components might prevent the formation of complete and functional signaling modules. Facilitation of Raf activation requires membrane localization of KSR (10, 12). Localization of an overexpressed KSR scaffold to the cytosol (Fig. 2) might also explain quenching of the ERK MAP kinase signal. Alternatively, a KSR scaffold could incorporate phosphatases or other molecules that deactivate other components of the module (28).

Taken together with previous data, these results support the possibility that KSR could function to modulate the duration and intensity of ERK MAP kinase stimulation. The magnitude of ERK MAP kinase activation controls its subcellular distribution (29, 30) and is the basis for a model that explains how
the activation of the same signaling pathway by different growth factor receptors can result in conspicuous differences in cell proliferation and differentiation (31). The data presented here provide experimental evidence and a rationale for the analysis of KSR as an unique modulator of ERK MAP kinase activation with implications for normal and abnormal cell growth and development.

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Note Added in Proof—While this manuscript was under review, papers were published that also demonstrated the ability of KSR to inhibit ERK MAP kinase activity (32) and Ras-induced transformation (33).

REFERENCES
1. Schlessinger, J., and Bar-Sagi, D. (1994) Cold Spring Harbor Symp. Quant. Biol. 59, 173–180
2. White, M. A., Nicotella, C., Minden, A., Polverino, A., Van Aelst, L., Karin, M., and Wigler, M. H. (1995) Cell 80, 533–541
3. Rodriguez-Viciana, P., Warne, P. H., Dhand, R., Vanhaesebroeck, B., Gout, J., Fry, M. J., Waterfield, M. D., and Downward, J. (1994) Nature 370, 527–532
4. Van Aelst, L., Barr, M., Marcus, S., Polverino, A., and Wigler, M. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 6213–6217
5. Marshall, C. J. (1995) Cell 10, 2684–2695
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