PHOSPHORYLATION REGULATES KSR1 STABILITY, ERK ACTIVATION, AND CELL PROLIFERATION

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Running Title: KSR1 phosphorylation regulates ERK signaling

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Summary

Kinase Suppressor of Ras (KSR) is a molecular scaffold that interacts with the components of the Raf/MEK/ERK kinase cascade and positively regulates ERK signaling. Phosphorylation of KSR1, particularly at Ser\(^{392}\), is a critical regulator of KSR1 subcellular localization and ERK activation. We examined the role of phosphorylation of both Ser\(^{392}\) and Thr\(^{274}\) in regulating ERK activation and cell proliferation. We hypothesized that KSR1 phosphorylation is involved in generating signaling specificity through the Raf/MEK/ERK kinase cascade in response to stimulation by different growth factors. In fibroblasts, PDGF stimulation induces sustained ERK activation and promotes S-phase entry. Treatment with EGF induces transient ERK activation, but fails to drive cells into S phase. Mutation of Ser\(^{392}\) and Thr\(^{274}\) (KSR1.TVSA) promotes sustained ERK activation and cell cycle progression with either PDGF or EGF treatment. KSR1\(^{-/-}\) mouse embryo fibroblasts expressing KSR1.TVSA proliferate two times faster and grow to a higher density than cells expressing the same level of wild-type KSR1. In addition, KSR1.TVSA is more stable than wild-type KSR1. These data demonstrate that phosphorylation and stability of the molecular scaffold KSR1 are critical regulators of growth-factor specific responses that promote cell proliferation.
Introduction

Specific signal transduction pathways in mammalian cells are potent regulators of mitogenesis. Aberrant regulation of such pathways can lead to oncogenesis. The proto-oncogene Ras is a small GTPase that couples signals from extracellular stimuli to intracellular pathways controlling cell growth, differentiation, and survival (1-3). Activation of the Raf-MEK1-ERK (p42/p44) MAP kinase cascade by Ras modulates cell growth, differentiation, and survival (4-6).

The Ras-Raf/MEK/ERK pathway mediates signals downstream of a variety of extracellular stimuli. Distinct responses to different stimuli can be generated by modulating the duration of ERK activation. In PC12 cells, NGF induces sustained ERK activation to promote neuronal cell differentiation, whereas EGF induces transient ERK activation to promote cell proliferation (7,8). In contrast, EGF induces transient ERK activation in fibroblasts to promote survival, but not mitogenesis, while PDGF induces sustained ERK activation and promotes S phase entry and cell proliferation (7,9). It is unclear, however, how different extracellular ligands differentially regulate the duration of ERK activation. Different adaptors may be recruited to the PDGF and EGF receptors, which may change the duration or intensity of the signal directed at the Raf/MEK/ERK kinase cascade. As an alternate mechanism, molecular scaffolds regulate the intensity and duration of signaling output in a concentration-dependent manner (10-15). Molecular scaffolds may also regulate the interaction, subcellular localization, or stability of the signaling components that they bind.

The protein Kinase Suppressor of Ras (KSR1) is a molecular scaffold and positive effector for the Raf/MEK/ERK kinase cascade (16-18). KSR1 was originally identified in
Caenorhabditis elegans and Drosophila melanogaster as a modifier of activated Ras (19-21). KSR1 associates with Raf, MEK, and ERK (22-28) and positively regulates ERK activation (16,18,22,23,29). KSR1 is phosphorylated on multiple sites by associated kinases (30,31). KSR1 has been reported to be phosphorylated on Ser$^{392}$ by both C-TAK1 and nm23-H1 (32,33). Phosphorylation of Ser$^{392}$ regulates the subcellular distribution of KSR1 (32,34,35) and generates the major 14-3-3 binding site on KSR1 (30,32). Dephosphorylation of KSR1 on Ser$^{392}$ by PP2A allows the translocation of KSR1 from the cytosol to the plasma membrane, where it facilitates the activation of MEK by Raf (35). Consequently, the dephosphorylation of Ser$^{392}$ on KSR1 promotes ERK activation. Given its role in ERK activation, we hypothesized that phosphorylation of Ser$^{392}$ on KSR1 could modify the duration of ERK activation and, as a consequence, the mitogenic potential of growth factors.

Phosphorylation of three residues, Thr$^{274}$, Ser$^{260}$, and Ser$^{443}$, is inducible by pathway stimulation with growth factors or activated Ras (30). The amino acids surrounding each of these phosphorylation sites is a consensus MAPK phosphorylation sequence (30). Interestingly, Thr$^{274}$ appears to be phosphorylated in response to treatment with PDGF (30), but not EGF (32). We hypothesized that Thr$^{274}$ phosphorylation may be a mechanism by which KSR1 promotes the mitogenic action of PDGF and restricts that mitogenic action of EGF.

In this study, we demonstrate that the phosphorylation of KSR1 at residues Thr$^{274}$ and Ser$^{392}$ modulates the proliferative potential of specific growth factors. When expressed at levels observed in wild type fibroblasts, ectopic KSR1 restored sustained ERK activation upon PDGF stimulation, and transient ERK activation upon EGF stimulation. However similar expression of KSR1 mutated at phosphorylation sites Thr$^{274}$ or Ser$^{392}$ promoted sustained ERK activation in response to stimulation with both EGF and PDGF and accelerated cell proliferation. Therefore,
blocking KSR1 phosphorylation generated a PDGF-like response to EGF treatment, suggesting that KSR1 phosphorylation mediates ERK signaling specificity to EGF and PDGF stimulation. In combination, these mutations also inhibited KSR1 turnover. These data suggest that proliferative responses through the Raf/MEK/ERK signaling cascade can be regulated by controlling the phosphorylation and stability of KSR1.
Experimental Procedures

Cell culture. 293T cells and immortalized KSR1−/− mouse embryo fibroblasts (MEF) were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (Atlanta Biologicals), 1% penicillin/streptomycin, 100 nM MEM non-essential amino acids, and 2 mM L-glutamine, and were incubated at 37°C in a 6% CO2 atmosphere. KSR1−/− MEFs were generated from day 13.5 KSR1−/− embryos and were immortalized by a 3T3 protocol as described previously (16).

Generation of stable cell lines. The bicistronic KSR1 construct was generated by subcloning mouse KSR1-Flag (36) or mutated forms of KSR1 (31,34) into MSCV-IRES-GFP using EcoRI and Sall/XhoI restriction endonuclease sites. KSR1 retroviruses were generated by cotransfecting MSCV-KSR1-IRES-GFP retroviral expression plasmids with an ecotropic packaging vector into 293T cells (45). KSR1−/− MEFs were infected by incubating with the retroviral supernatant for 24 hours in the presence of 4 µg/ml polybrene. The pool of infected cells was sorted by FACS analysis, and the population of cells expressing the lowest 10% of GFP levels, and therefore the lowest 10% of KSR1 levels, was collected (18). Cells were excited at 458 nm and separated at 510/20 nm, with baseline fluorescence of uninfected cells having a mean intensity of 6 (range 0-15). Post-sorted cells were assessed for purity by FACS analysis. KSR1 expression was confirmed by western blot.

Immunoprecipitation and Western blotting. Cells were lysed in ice-cold lysis buffer (0.5% Triton X-100, 120 mM NaCl, 40 mM Tris-HCl [pH 7.4], 10 mM NaPPi, 2 mM EGTA, 2 mM EDTA, 10 mM NaF, 10 µg/ml leupeptin, 5 µg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, 2 mM sodium vanadate). Clarified cell lysates were normalized for total protein...
concentration using the BCA protein assay (Pierce). Proteins were resolved by SDS-polyacrylamide gel electrophoresis on 10% gels and were transferred to nitrocellulose membranes (Amersham). The membranes were probed with antibodies against β-actin (Santa Cruz), KSR1 (BD Biosciences), ERK/phosphoERK Thr<sup>202</sup>/Tyr<sup>204</sup> (Santa Cruz and Cell Signaling Technology), RSK/phosphoRSK Ser<sup>380</sup> (BD Biosciences and Cell Signaling Technology), or MEK/phosphoMEK Ser<sup>217</sup>/Ser<sup>221</sup> (BD Biosciences and Cell Signaling Technology). Western blots were probed with goat anti-mouse or anti-rabbit secondary antibodies conjugated to AlexaFluor 680 goat anti-mouse IgG (Molecular Probes) or IRdye 800 (Rockland Immunochemicals). Blotted proteins were detected and quantified using the Odyssey infrared imaging system (LI-COR).

**ERK Timecourses.** Timecourses of ERK activation were performed using the In-Cell Western blot analysis on the Odyssey system (LI-COR), which allows analysis and quantification of ERK phosphorylation in multiple samples by simultaneously probing fixed cells with antibodies against total ERK and ERK phosphorylated on Thr<sup>202</sup> and Tyr<sup>204</sup>. KSR<sup>1</sup>−/− MEFs expressing KSR1 constructs were seeded in triplicate at 1x10<sup>5</sup> cells/well in a 96-well plate (BioSource International, Inc.). Twenty-four hours after plating, cells were serum-deprived for four hours and stimulated with EGF (100 ng/ml) or PDGF-BB (25 ng/ml) for the indicated timepoints. Cells were fixed in 3.7% formaldehyde for 20 min. at room temperature and stained for ERK/phosphoERK (Santa Cruz sc-94, 1:200, Cell Signaling Technology 9106, 1:200) according to manufacturers’ instructions and as described previously (18). AlexaFluor 680 anti-rabbit IgG (Molecular Probes, 1:200) and IRDye 800 anti-mouse IgG (Rockland Immunochemicals, 1:200) were used as secondary antibodies. Plates were scanned using the...
Odyssey system. Results were analyzed using the In-Cell Western Blot Odyssey software (LI-COR) and Microsoft Excel.

**Proliferation Assays and BrdU incorporation.** To assay growth rate, cells were seeded in a 12-well plate at 2.5 x 10^4 cells per well. Cells from triplicate wells were trypsinized, harvested, and counted each day for six days using a Beckman Coulter Counter.

Cells were assayed for cell cycle progression by monitoring incorporation of 5-bromo-2-deoxyuridine (BrdU, BD Biosciences). Cells were plated at 1.6x10^4 cells per well on coverslips. Four hours after plating, cells were serum-starved for 72 hours to induce quiescence. Cells were then stimulated with EGF (100 ng/ml), PDGF-BB (25 ng/ml), or serum (10% fetal bovine serum) in the presence of BrdU (10 μM) for 20 hours. The cells were fixed in methanol:acetone (1:1) at -20ºC for 8 min and were rehydrated with TBS for 10 min at room temperature. After treatment with 1.5 M HCl for 10 minutes, cells were blocked with Odyssey blocking buffer (LI-COR) for 40 minutes and incubated for 40 min with anti-BrdU antibody conjugated to phycoerythrin (PharMingen Becton Dickinson, 1:1 with Odyssey blocking buffer). Nuclei were stained with Hoechst dye 33258. Cells were visualized on a fluorescence microscope with the appropriate filters.

**Transformation Assays.** 5x10^3 cells were resuspended in 0.325% Noble Agar (in Iscove’s Modified Dulbecco’s Medium, 15% FBS, 1% penicillin/streptomycin, and 2 mM L-glutamine) and plated on a layer of 0.65% Noble Agar. Colonies were counted after three weeks.

**Protein Turnover.** KSR1−/− MEFs expressing ectopic KSR1 constructs were serum-starved in the presence of cycloheximide (50 μg/ml, Sigma) for 0-8 hours. Cells were snap-frozen and lysed in NP40 lysis buffer (20 mM Tris-HCl [pH 8.0], 137 mM NaCl, 10% glycerol,
1% IGEPAL, 1 mM phenylmethylsulfonyl fluoride, 5 mM sodium vanadate, 5 µg/ml aprotinin, and 10 µg/ml leupeptin). Protein levels were determined by western blotting with antibodies against KSR1 and actin and were quantified using Odyssey software (LI-COR).
Results

Phosphorylation of KSR1 modulates the duration of ERK activation. KSR1 expression promotes ERK activation (18). We hypothesized that phosphorylation of KSR1 contributes to its role in ERK activation by EGF and PDGF. To investigate this possibility, we expressed wild-type and mutated forms of KSR1 in KSR1⁻/⁻ MEFs (16) at levels comparable to those in wild-type MEFs (Fig. 1).

KSR1⁻/⁻ MEFs expressing ectopic KSR1 exhibited sustained ERK activation with PDGF stimulation (Fig. 2A). ERK activation peaked at 5 minutes after treatment, but showed significant phosphorylation out to 90 minutes of stimulation. In contrast, upon treatment with EGF, the cells exhibited transient ERK activation (Fig. 2B), with ERK phosphorylation peaking at 5 minutes of stimulation but falling rapidly back to basal levels.

We examined the effects of mutating Thr²⁷⁴ or Ser³⁹² (Thr²⁷⁴ to Val, TV; Ser³⁹² to Ala, SA) on the time course of ERK activation upon stimulation with both EGF and PDGF. Mutation of Ser³⁹² on KSR1 promotes KSR1 membrane recruitment and Xenopus oocyte maturation (32). KSR1.SA caused sustained ERK activation in cells treated with PDGF (Fig. 2A). In cells treated with EGF, KSR1.SA promoted a burst of ERK activation that was sustained above the level obtained in cells expressing wild-type KSR1 (Fig. 2B). These data suggest that Ser³⁹² dephosphorylation enhances both the intensity and duration of ERK activation. ERK activation was decreased in both intensity and duration in KSR1⁻/⁻ MEFs expressing KSR1.TV upon treatment with either PDGF (Fig. 2A) or EGF (Fig. 2B). These data demonstrate that blocking phosphorylation of this putative ERK phosphorylation site alone does not facilitate ERK phosphorylation.
To determine which KSR1 mutation was dominant (SA or TV), we expressed KSR1 mutated at both Thr\(^{274}\) and Ser\(^{392}\) (TVSA) in KSR1\(^{-/-}\) MEFs (Fig. 1). Cells expressing KSR1.TVSA demonstrated more sustained ERK activation upon PDGF stimulation (Fig. 2A) compared to wild-type KSR1 expressed at the same level. Cells expressing KSR1.TVSA also exhibited markedly prolonged ERK activation upon stimulation with EGF (Fig. 2B). Therefore, the prolonged ERK activation generated by KSR1.SA was dominant to the decreased ERK activation induced by KSR1.TV. Furthermore, combined mutation of Ser\(^{392}\) and Thr\(^{274}\) synergistically promoted sustained ERK activation upon treatment with either EGF or PDGF. These data suggest that the phosphorylation of KSR1 on Thr\(^{274}\) and Ser\(^{392}\) can modulate the time course of ERK activation in response to specific growth factors.

**Phosphorylation of KSR1 modulates S-phase entry.** In fibroblasts, sustained ERK activation allows quiescent cells to re-enter the cell cycle, whereas transient ERK activation does not (7,9). The phosphorylation of Thr\(^{274}\) and Ser\(^{392}\) on KSR1 is important for modulating the duration of ERK activation (Fig. 2A and 2B). Therefore, the ability of KSR1 phosphorylation to regulate S-phase entry was measured by BrdU incorporation into DNA.

In comparison to untreated cells, fetal bovine serum and PDGF, but not EGF, were sufficient to promote S-phase entry in at least 90% of cells expressing ectopic KSR1 (Figs. 3 and S1). Both KSR1.SA and KSR1.TV were similarly competent to promote cell cycle progression in the presence of serum. KSR1.SA expression caused S phase entry in 85% of cells treated with PDGF and in 40% of cells treated with EGF. In comparison to control cells, EGF and PDGF had minimal ability to induce S phase entry in cells expressing KSR1.TV (Fig. 3).

To further investigate the role of KSR1 phosphorylation in mediating cell cycle progression, we assayed S-phase entry in cells expressing KSR1.TVSA. PDGF treatment of
KSR1−/− MEFs expressing KSR1.TVSA was sufficient to promote cell-cycle progression (Figs. 3 and S1). Upon expression of KSR1.TVSA, EGF treatment also induced S phase entry in 76% of cells. Thus, combined mutation of Ser392 and Thr274 in KSR1 conferred a PDGF-like response to cells treated with EGF. These data indicate that KSR1 phosphorylation can modulate growth factor-induced cell cycle progression by regulating the duration of ERK activation.

**KSR1 phosphorylation regulates cell proliferation.** To determine if KSR1 phosphorylation also regulates mitogenesis in cultured cells, we assessed proliferation rates of KSR1−/− MEFs expressing KSR1 or KSR1 phosphorylation mutants. KSR1.SA induced a modest increase in proliferation rate compared to wild-type KSR1 (Fig. 4A). However, cells expressing KSR1.TVSA showed a two-fold increase in proliferation rate during log-phase growth compared to wild-type KSR1, and grew to an increased density (Fig. 4A). Expression of KSR1.TV alone slowed proliferation slightly in comparison to wild-type KSR1, consistent with its inhibitory effects on ERK activation. These data indicate that the phosphorylation of KSR1 on Ser392 and Thr274 are important regulators of a cell’s proliferative potential.

**KSR1 phosphorylation regulates oncogenic transformation.** KSR1−/− MEFs are resistant to oncogenic transformation by activated RasV12, but reintroduction of wild type KSR1 rescues the transformation defect in a dose-dependent manner (18). To determine if KSR1 phosphorylation also regulates cell transformation, we assayed anchorage-independent growth of MEFs on soft agar. When expressed in KSR1−/− MEFs at levels comparable to those observed in wild-type MEFs, neither ectopic KSR1 nor KSR1.TVSA allowed growth in soft agar. These data suggest that the increased proliferative potential associated with KSR1.TVSA is not sufficient to induce cell transformation (Fig. 4B). We also generated MEFs expressing increasing amounts of KSR1 or KSR1.TVSA by sorting and selecting cells expressing increasing
levels of GFP from the bicistronic vector. Overexpression of wild-type KSR1 at any level was not sufficient to promote cell transformation. However, moderate overexpression of KSR1.TVSA induced the anchorage-independent growth of KSR1−/− MEFs, even in the absence of activated RasV12 (Fig. 4B). Increasing expression of KSR1.TVSA inhibited cell transformation to basal levels, as observed previously in cells overexpressing wild type KSR1 (18).

**KSR1 phosphorylation does not regulate the duration of MEK activation.** The phosphorylation of Ser392 contributes to the activation of MEK by Raf (32). To determine whether prolonged ERK activation in cells expressing KSR1.TVSA was a result of the mutated scaffold’s effect on MEK, we characterized the activation of MEK1/2 in response to PDGF and EGF. KSR1−/− MEFs expressing ectopic KSR1 or KSR1.TVSA were serum-starved and stimulated with EGF or PDGF for 0, 5, 30, or 120 minutes. In cells expressing KSR1.TVSA, MEK phosphorylation was not prolonged by PDGF or EGF. Though MEK phosphorylation was elevated 5 minutes after EGF treatment, it was reduced back to basal levels by 30 minutes, comparable to wild-type KSR1 (Fig. 5A). These data show that the prolonged activation of ERK by KSR1.TVSA does not result from the prolonged phosphorylation and activation of MEK.

We next assayed the phosphorylation and activation of the ERK substrate p90 RSK1 by western blot. As a direct consequence of sustained ERK activation, the phosphorylation of the ERK substrate p90 RSK1 is also prolonged 120 minutes after EGF stimulation in the presence of KSR1.TVSA, compared to wild-type KSR1 (Fig. 5B). These data demonstrate that KSR1 phosphorylation, by mediating the duration of ERK activation, also regulates the activation of downstream targets of the Raf/MEK/ERK cascade. In addition to showing prolonged activation, the levels of total RSK1 are elevated in cells expressing KSR1.TVSA. Increased expression of
p90 RSK1, a cyclin-responsive gene, is attributable to the elevated cyclin D1 activity associated with the increased proliferative rate of cells expressing KSR1.TV.S932A (37).

**Phosphorylation of KSR1 modulates KSR1 stability.** The expression level of the molecular scaffold KSR1 regulates its biological effects (18,24,30). Overexpression of KSR1 inhibits ERK activation and ERK-induced phenotypes (24,25,36). However, careful titration of KSR1 levels reveals that increasing the ectopic expression of KSR1 in KSR1−/− MEFs up to 14-fold above levels observed in wild-type MEFs promotes sustained ERK activation and mitogenesis (18). Similarly, expression of KSR1.TVSA allowed sustained ERK activation and promoted cell cycle progression and cell proliferation (Figs. 2-4). We hypothesized that mutation of Thr274 and Ser392 in MEFs mimicked the effects of moderate overexpression of KSR1 by increasing the stability of the scaffold.

To determine if KSR1 turnover rate was altered by the mutation of Thr274 and Ser392, KSR1−/− MEFs expressing ectopic KSR1 or KSR1.TVSA were serum-starved in the presence of cycloheximide (50 µg/ml) for 0-8 hours. The half-life of KSR1 in serum-free medium was approximately 1 hour, whereas the half-life of KSR1.TVSA was approximately 6 hours (Fig. 6A). Mutation of Thr274 or Ser392 individually was not sufficient to increase the stability of KSR1 (Fig. 6B). These data demonstrate that KSR1.TVSA is more stable than wild-type KSR1, and suggest that phosphorylation of KSR1 on Thr274 and Ser392 modulates KSR1 turnover and degradation.
Discussion

Data shown here demonstrate that cells can control their responses to growth factor stimulation through phosphorylation of the molecular scaffold KSR1. Mutations in KSR1 that impair phosphorylation of Thr\textsuperscript{274} and Ser\textsuperscript{392} alter the biochemical and biological outcome of MAP kinase pathway signaling. Combined mutation of Thr\textsuperscript{274} and Ser\textsuperscript{392} conferred a PDGF-like response to EGF treatment. KSR1.TVSA induced sustained ERK activation and promoted cell cycle progression upon treatment with either PDGF or EGF. Cells expressing KSR1.TVSA also proliferated at an increased rate, and elevated KSR1.TVSA expression caused cell transformation. In addition, KSR1.TVSA was more stable than wild-type KSR1, suggesting that the phosphorylation of KSR1 regulates its turnover and, in turn, regulates the duration of ERK activation.

The duration of ERK signaling is critical to generating specific biological responses to MAP kinase pathway signaling. Prolonged ERK activation promotes its sustained nuclear localization, stabilization of immediate early gene products, and cell-cycle progression (7,9,38). To understand how KSR1 mutations that alter its phosphorylation may affect ERK activation, we studied the time course of ERK activation in cells expressing wild type or mutated KSR1. Cells expressing KSR1.SA showed robust, and somewhat prolonged, ERK activation upon treatment with EGF. Expression of KSR1.SA also induced a modest increase in proliferation rate compared to wild-type KSR1 (Fig. 4A), and modestly increased S-phase entry upon EGF stimulation (Fig. 3). This is consistent with data in Cos cells demonstrating that mutation of Ser\textsuperscript{392} to Ala promotes MEK activation by Raf (32). Dephosphorylation of Ser\textsuperscript{392} by PP2A is required for KSR1 release from 14-3-3 and translocation to the plasma membrane (35). Thus,
KSR1.SA is already primed for membrane localization in the absence of a mitogenic signal, which explains how KSR1.SA promotes ERK activation and cell cycle progression.

Mutation of Thr274 on KSR1 inhibited ERK activation in both intensity and duration in response to treatment with either EGF or PDGF (Fig. 2). As a consequence, mutation of Thr274 alone was not sufficient to promote cell proliferation (Fig. 4A) or cell cycle progression upon EGF or PDGF treatment (Fig. 3). However, the combined mutation of Ser392 and Thr274 induced sustained ERK activation upon treatment with either PDGF or EGF, promoted S-phase entry with either PDGF or EGF treatment (Fig. 3), and caused an increased rate of proliferation compared to cells expressing wild-type KSR1 (Fig. 4A). These data suggest that the dynamic phosphorylation and dephosphorylation of Ser392 and Thr274 work together to promote mitogenesis by regulating the duration of ERK activation to generate signaling specificity. The data indicate that, if Ser392 is dephosphorylated, dephosphorylation of Thr274 (or blocking its phosphorylation) can serve as an activating step in ERK signaling and is critical for determining the duration of ERK activation.

The kinases C-TAK and nm23-H1 both interact with and phosphorylate KSR1 on Ser392 (32,33). The kinase that phosphorylates KSR1 on Thr274 in intact cells is not known. However, Thr274 resides in a consensus sequence for phosphorylation by ERKs and the MEK inhibitor PD98059 inhibits Thr274 phosphorylation (30). The KSR1 kinase domain could autophosphorylate on Thr274. Though the kinase domain of mouse and human KSR1 lack evolutionarily conserved sequences that are typically required for catalytic activity (21), KSR1 has been reported to be an active kinase (39-42). However, other evidence demonstrates that KSR1 function is independent of the kinase domain (26,29,32). Autophosphorylation on Thr274
is unlikely, since KSR1 constructs that lack the entire KSR1 kinase domain are still phosphorylated on Thr^{274} in immune complex kinase assays (31).

Cells expressing KSR1.TVSA are able to interpret signals induced by both PDGF and EGF binding as mitogenic. Consequently, cells expressing KSR1.TVSA may interpret more growth factor-induced signals as mitogenic than do cells expressing wild type KSR1. We believe this hyper-mitogenic signaling accounts for the increased proliferation of MEFs in culture expressing KSR1.TVSA.

A current model for KSR1 function in cells is that KSR1 brings MEK to Raf to be activated at the plasma membrane (32). Based on these observations, we hypothesized that KSR1 regulated the duration of ERK activation by regulating the duration of MEK activation. In cells expressing KSR1.TVSA, we observed that MEK phosphorylation upon EGF treatment is slightly elevated compared to cells expressing wild-type KSR1. However, phospho-MEK levels were not sustained following EGF treatment compared to wild-type KSR1 (Fig. 5A). While KSR1 may facilitate the phosphorylation of MEK by Raf, our data suggest that KSR1 phosphorylation may also regulate ERK activation independent of its effects on MEK.

The expression of KSR1 is required for the complete activation of the Raf/MEK/ERK kinase cascade. Deletion of KSR1 impairs growth factor-induced activation of ERK and prevents the transforming effects of activated Ras^{V12} in MEFs. Expression of ectopic KSR1 into KSR1^{-/-} MEFs at levels comparable to those found in wild-type MEFs restores ERK activation and Ras^{V12}-induced cell transformation without any effects on cell proliferation in the absence of an oncogene (18). In an experimental system, overexpression of KSR1 to levels that interact optimally with Raf, MEK and ERK is sufficient to enhance ERK activation and cell proliferation. Altered levels of KSR expression may play a role in regulating cell
responsiveness. KSR1 is up-regulated during 1,25-dihydroxyvitamin D3-mediated
differentiation of HL60 cells (43) and following NGF-induced differentiation of PC12 cells (26).
However, overexpression of a scaffold protein beyond a stoichiometric optimum results in the
titration of its binding partners apart from each other and inhibits the biologic activity of its
kinase cascade (10,44,45). These data suggest that the expression of a molecular scaffold such
as KSR1 is a potential point of regulation in a cell to modulate the biochemical and biological
output of a signaling cascade. Here we show that KSR1.TVSA has increased stability compared
to wild-type KSR1 (Fig. 6), suggesting that phosphorylation of KSR1 at Thr$^{274}$ and Ser$^{392}$
negatively regulates KSR1 protein stability. In addition, KSR1.TVSA allowed sustained ERK
activation upon EGF stimulation and consequently enhanced cell proliferation. The increased
stability of KSR1.TVSA may promote sustained ERK activation by facilitating more productive
interactions between phospho-MEK and inactive ERK, or by increasing the pool of KSR1
available for recruitment to a signaling complex. It is also possible that the increased pool of
KSR1 binds and stabilizes phospho-ERK. KSR1 may protect ERK from dephosphorylation by
MAP kinase phosphatases, either by steric regulation or by promoting ERK localization to an
optimal site of action.

The mechanism for regulation of stability of KSR1 through phosphorylation has not been
elucidated. While KSR1 transcript levels are high in all tissues except liver, KSR1 protein
expression is only detectable in the brain, bladder, ovary, testis, and lung (46), suggesting the
presence of a mechanism regulating KSR1 translation or stability. KSR1 binds the chaperone
proteins Hsp90, Hsp70, and Hsp68 (26). These interactions may stabilize KSR1 protein
expression. Treatment of cells overexpressing KSR1 with geldanamycin to inhibit Hsp activity
increases the turnover of KSR1, either through a direct or indirect mechanism (26). It is possible
that altering the phosphorylation state of KSR1 modifies its association with heat shock proteins, thereby regulating its stability. In addition, KSR1 has been shown to associate with the E3 ubiquitin ligase IMP (47), but IMP-mediated ubiquitination of KSR1 has not been detected.

Phosphorylation of KSR1 on Ser$^{392}$ or Thr$^{274}$ may stimulate the turnover of KSR1 by promoting interaction with a ubiquitin ligase or by inhibiting interaction with a stabilizing protein. Based on its surrounding sequence, Thr$^{274}$ is a putative MAP kinase phosphorylation site (30). Thr$^{274}$ phosphorylation by active ERK might serve as a potential site of feedback regulation promoting the interaction of KSR1 with a destabilizing protein or dissociating KSR1 from a stabilizing protein.

Interestingly, increasing the expression level of KSR1.TVSA stimulated anchorage-independent growth in soft agar, a hallmark of cell transformation (Fig. 4B). It has been shown previously that KSR1 is required for transformation induced by expression of Ras$^{V12}$ (18,48), but expression of wild-type KSR1 at any level is not sufficient to induce cell transformation on its own (Fig. 4B). Moderate overexpression of KSR1.TVSA promoted cell transformation, even in the absence of an oncogene. These data demonstrate that KSR1 phosphorylation is a potent regulator of a cell’s proliferative and oncogenic potential. Moreover, the data suggest that anchorage-independent growth may require a sustained level of signal output from the Raf/MEK/ERK signaling cassette that can be attained by the combination of prolonged ERK activation provided by KSR1.TVSA with elevated levels of scaffold expression. The ability of KSR1 to function as an oncogene is dependent upon a defined level of expression. KSR1.TVSA fails to support anchorage-independent growth when expressed at high levels. Similarly, overexpression of wild type KSR1 at comparable levels suppresses colony formation induced by oncogenic Ras$^{V12}$ (18). This concentration-inhibitory effect of wild type KSR1 to inhibit Ras$^{V12}$-
induced cell transformation occurs at levels of the scaffold that exceed those required for optimal interactions with Raf, MEK and ERK (18). Thus, the transforming potential of KSR1.TVSA remains subject to, and limited by, its function as a scaffold.

A GFP fusion protein of KSR1 cycles through the nucleus in a phosphorylation dependent manner (34). GFP-KSR1 is primarily cytoplasmic in quiescent cells. However, mutation of phosphorylation sites Thr274 and Ser392 causes the redistribution of GFP-KSR1 to the nucleus. The biological significance of KSR1’s nuclear localization and the regulation of its subcellular trafficking remain to be characterized. However, data shown here suggest that nuclear localization of KSR1 may be a function of KSR1 stability and the duration of activity by the Raf/MEK/ERK kinase cascade.

The molecular scaffold KSR1 is a dynamic effector of signaling through the Raf/MEK/ERK kinase cascade. Its function is modified by its phosphorylation state and is subject to input from multiple pathways. These observations suggest that KSR1 may serve as an excellent control point for receiving input from intracellular mechanisms that positively or negatively regulate mitogenic potential and other aspects of cell fate affected by the Raf/MEK/ERK kinase cascade.

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Footnotes:
1. The abbreviations used are: MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; ERK, extracellular-regulated kinase; MAPK, mitogen-activated protein kinase; KSR1, kinase suppressor of Ras; EGF, epidermal growth factor; PDGF, platelet-derived growth factor; MEF, mouse embryo fibroblast; IRES, internal ribosomal entry site; KSR1.TV, KSR1 mutated from Thr to Val at position 274; KSR1.SA, KSR1 mutated from Ser to Ala at position 392; KSR1.TVSA, KSR1 mutated from Thr to Val at position 274 and from Ser to Ala at position 392; C-TAK1, cdc25c-associated kinase; Hsp, heat shock protein.

2. G. Razidlo and R. Lewis, unpublished data.
Figure Legends

**FIG. 1.** *Generation of KSR1-expressing cells.* KSR1<sup>−/−</sup> MEFs were infected with a bicistronic retroviral vector encoding GFP and epitope-tagged KSR1, KSR1.TV, KSR1.SA, or KSR1.TVSA as described in *Experimental Procedures.* Proteins were resolved by SDS-PAGE and immunoblotted with an anti-KSR1 antibody to verify KSR1 expression. The expression level of each KSR1 construct was compared to the endogenous level of KSR1 expressed in KSR1<sup>+/+</sup> MEFs.

**FIG. 2.** *Mutation of KSR1 phosphorylation sites enhances the duration of ERK activation.* KSR1<sup>−/−</sup> MEFs expressing KSR1, KSR1.TVSA (closed and open circles, respectively; left panels) KSR1.TV, or KSR1.SA (open and closed triangles, respectively; right panels), were analyzed in an in-cell western blot as described in *Experimental Procedures* for ERK activation. The cells were probed for total ERK and phosphorylated ERK in triplicate. Quantification of phosphorylated ERK normalized to total ERK is plotted versus time after stimulation by PDGF (A) and EGF (B).

**FIG. 3.** *Mutation of KSR1 phosphorylation sites regulates S-phase entry.* Quiescent KSR1<sup>−/−</sup> MEFs expressing ectopic KSR1, KSR1.TV, KSR1.SA, or KSR1.TVSA were treated with EGF, PDGF or serum for 20 hours in the presence of BrdU. DNA synthesis was detected by immunofluorescence using an anti-BrdU antibody and by counterstaining all nuclei with DAPI. More than 100 cells were scored in each experiment to determine the percentage of BrdU-positive nuclei. The data represent mean values ± standard deviation for three independent trials.
**Fig. 4.** 
**KSR1 phosphorylation regulates cell proliferation and transformation.** (A) KSR1<sup>−/−</sup> MEFs expressing ectopic KSR1 (closed circles), KSR1.TV (open triangles), KSR1.SA (closed triangles), or KSR1.TVSA (open circles) were seeded at 25,000 cells per well. Cells in triplicate wells were isolated by trypsin digestion on successive days after plating and counted using a Beckman Coulter Counter. Cell numbers are plotted as the average ± standard deviation of three trials. (B) KSR1<sup>−/−</sup> MEFs expressing ectopic KSR1 or KSR1.TVSA at increasing levels were plated in soft agar to assess anchorage-independent growth. Colonies were grown for three weeks and then counted. Numbers of colonies (per 5000 cells plated) are plotted as the average ± the standard deviation of three trials.

**Fig. 5.** 
**The effect of KSR1 phosphorylation on MEK1/2 and RSK1 activation.** (A) KSR1<sup>−/−</sup> MEFs expressing KSR1 or KSR1.TVSA were treated with EGF or PDGF for the indicated times and lysates were resolved by SDS-PAGE. Samples were immunoblotted with the antibodies against phospho-MEK and total MEK. Representative western blots were quantified using Odyssey software (LI-Cor). Phospho-MEK levels were normalized to total MEK and plotted as the fold induction of MEK phosphorylation compared to unstimulated cells. (B) Cells treated as described in (A) were analyzed by western blot using antibodies against p90 RSK and phospho-RSK. Phospho-RSK levels were normalized to total RSK and plotted as the fold induction of RSK phosphorylation compared to unstimulated cells.

**Fig. 6.** 
**Phosphorylation regulates KSR1 stability.** KSR1<sup>−/−</sup> MEFs expressing ectopic KSR1 constructs were treated with cycloheximide in serum-free medium for the indicated times. Cell lysates were analyzed by western blot using an anti-KSR1 antibody. KSR1 levels were
quantified using Odyssey software and were normalized to actin levels at each timepoint. Data are represented as the percentage of KSR1 present compared to untreated cells (t=0) in cells expressing (A) KSR1 (closed circles), KSR1.TVSA (open circles), (B) KSR1.SA (closed triangles), or KSR1.TV (open triangles). Data are representative of three independent experiments.

**Fig. S1.** Fluorescent photomicrographs of anti-BrdU- and DAPI-stained cells. Cells were treated and prepared as described in Fig. 3.
Figure 1
Figure 2

**A**

- **KSR1**
- **KSR1.TVSA**

**B**

- **KSR1.SA**
- **KSR1.TV**
Figure 4

A

![Graph showing the number of cells x 10^(-5) over time for different conditions: KSR, KSR1.TV, KSR1.SA, and KSR1.TVSA.](image)

- KSR
- KSR1.TV
- KSR1.SA
- KSR1.TVSA

Day
0, 1, 2, 3, 4, 5, 6, 7

B

![Bar graph showing the number of colonies for KSR1 and KSR1.TVSA.](image)

- KSR1
- KSR1.TVSA

Number of colonies
0, 40, 80, 120, 160, 200

KSR
Actin
Figure 5

A

KSR1

KSR1.TVSA

pMEK

MEK

0 5 30 120 5 30 120 0 5 30 120 5 30 120 (minutes)

EGF PDGF EGF PDGF

KSR1 KSR1.TVSA

0 2 4 6 8 10 12 14 16 (minutes)

pMEK/MEK Fold Induction

KSR1 KSR1.TVSA

EGF PDGF EGF PDGF

B

KSR1

KSR1.TVSA

pRSK

RSK

0 5 30 120 5 30 120 0 5 30 120 5 30 120 (minutes)

EGF PDGF EGF PDGF

pRSK/RSK Fold Induction

KSR1 KSR1.TVSA

EGF PDGF EGF PDGF
Figure 6

A

KSR1

KSR1.TVSA

KSR1

Actin

Time (hours) Starved + CHX

Fold decrease in KSR1 expression (vs. Actin)

B

KSR1.TV

KSR1.SA

KSR1

Actin

Time (hours) Starved + CHX

Fold decrease in KSR1 expression (vs. Actin)
Figure S1

|                | KSR1 | KSR1.TV | KSR1-SA | KSR1.TVSA |
|----------------|------|---------|---------|-----------|
| Anti-BrdU      | ![Image](image1.png) | ![Image](image2.png) | ![Image](image3.png) | ![Image](image4.png) |
| DAPI           | ![Image](image5.png) | ![Image](image6.png) | ![Image](image7.png) | ![Image](image8.png) |
| Anti-BrdU      | ![Image](image9.png) | ![Image](image10.png) | ![Image](image11.png) | ![Image](image12.png) |
| DAPI           | ![Image](image13.png) | ![Image](image14.png) | ![Image](image15.png) | ![Image](image16.png) |

EGF

PDGF
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