Epidermal Growth Factor Treatment Enhances the Kinase Activity of Kinase Suppressor of Ras*

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In Drosophila melanogaster and Caenorhabditis elegans, kinase suppressor of Ras (KSR) functions as a positive modulator of Ras-dependent signaling either upstream of or parallel to Raf. Attempts to characterize the biochemical and biological properties of mammalian KSR, however, have had limited success. Although some studies demonstrated a requirement of KSR kinase activity for its action, others indicated the kinase function of KSR is dispensable and suggested that KSR acts primarily as a scaffold protein. Interpretations of KSR function are further hampered by the lack of a standardized assay for its kinase activity in vitro. To address this issue, we established a two-stage in vitro kinase assay in which KSR never comes in contact with any recombinant kinases other than c-Raf-1. Using this assay, we show that KSR immunoprecipitated from quiescent COS-7 cells overexpressing Flag-tagged KSR was inactive, but its activity was rapidly and markedly induced upon epidermal growth factor treatment. Moreover, KSR-reconstituted mitogen-activated protein kinase activation was detected in KSR immunoprecipitates depleted of all contaminating kinases (c-Raf-1, MEK1, ERK2) by multiple high salt washes. Only full-length kinase-active KSR was capable of signaling c-Raf-1-dependent activity as kinase inactive and C- and N-terminal deletion mutants were without effect. Furthermore, endogenous KSR isolated from A431 cells, which contain high levels of activated EGF receptor, displays constitutively enhanced kinase activity. Hence, KSR kinase activity is not an artifact of overexpression but a property intrinsic to this protein. The recognition of EGF as a potent activator of KSR kinase activity and the availability of a well defined in vitro kinase assay should facilitate the definition of the function of KSR as a Ras-effector molecule.

Recent investigations have identified a new class of upstream signals necessary for normal development and/or oncosupression via Ras (1–6). One component, kinase suppressor of Ras (KSR),1 was originally identified in Drosophila melanogaster and Caenorhabditis elegans to function as a positive modulator of Ras-mitogen-activated protein kinase (MAPK) signaling either upstream of or parallel to Raf (1–3). The isolation of murine and human KSR homologs with a high level of sequence identity (1) suggests that KSR signaling is evolutionarily conserved. Consistent with this hypothesis, in C. elegans, KSR regulates Ras signaling of vulval development, a pathway initiated through the C. elegans homolog of the epidermal growth factor (EGF) receptor LET-23 (2, 3). Attempts to characterize the biochemical and cell biological properties of mammalian KSR, however, have yielded a confusing and often contradictory picture of the role of this protein in signal transduction.

Morrison and co-workers (7, 8) and Muslin and co-workers (9) like ourselves (10, 11) found that KSR complexes with and activates c-Raf-1 to enhance signaling through the MAPK cascade. This interaction is reported to be an essential requirement for Xenopus laevis oocyte maturation, cellular transformation, and ceramide signaling of apoptosis in BAD-expressing cells. In contrast, Williams and co-workers (12) and Eyache and co-workers (13) reported that KSR binds to and functionally inactivates MEK1, blocking signaling through MAPK and attenuating Ras-induced cellular transformation and serum-induced mitogenesis. In some instances, this may reflect context-dependent or cell type-specific Ras signaling. For instance, KSR transduces apoptotic signals in response to ceramide in COS-7 cells only if these cells express BAD, a member of the pro-apoptotic Bcl-2 family (11). Alternatively, the net outcome may reflect gene dosage. Expression of small amounts of KSR cooperates with Ras for Xenopus oocyte maturation (7) whereas high level expression results in inhibition of Ras-mediated oocyte maturation and R7 photoreceptor formation in the Drosophila eye (14).

There is also a disagreement as to whether the kinase domain of KSR is obligatory for the biological functions of KSR (8, 10, 11). While we suggested that KSR kinase activity is required for ceramide signaling of apoptosis through BAD (11) and Lewis and co-workers (15) demonstrated that the KSR kinase domain is necessary for the inhibitory effect of KSR on ERK/MAPK kinase activity in response to growth factors, other groups have been able to overexpress portions of the KSR protein and mimic the function of full-length KSR independent of the kinase domain (7, 8, 14, 16). These latter findings have been interpreted as evidence that the kinase function of KSR is dispensable under physiologic conditions.

The interpretation of KSR function is further hindered by the lack of a standardized assay for its kinase activity in vitro.

1 The abbreviations used are: KSR, kinase suppressor of Ras; KI-KSR, kinase-inactive KSR; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; MBP, myelin basic protein; EGF, epidermal growth factor; Ab, antibody; GST, glutathione S-transferase; PAGE, polyacrylamide gel electrophoresis.
We first reported that the kinase activity of KSR was required to signal c-Raf-1 activation in an *in vitro* reconstitution assay using recombinant c-Raf-1, MEK1, and ERK2/MAPK, employing myelin basic protein (MBP) phosphorylation as a readout (10). Based on the inability to detect KSR kinase activity in reconstitution assays and the fact that numerous kinases co-immunoprecipitated with KSR, other groups have proposed that the observed activity was not intrinsic to KSR but rather that the primary mode of KSR signaling is via protein-protein interaction (the Scaffolding Hypothesis). To address this issue directly, we have established a new two-stage *in vitro* assay for KSR activity in which KSR never comes in contact with any recombinant kinases other than c-Raf-1. We demonstrate that extensive washing of the KSR immunoprecipitate with high salt (1 M NaCl) removes contaminating kinases but retains KSR activity. Using this assay, we show that Flag-KSR is virtually inactive in resting COS-7 cells, but its activity is markedly increased by EGF treatment. Furthermore, endogenous KSR isolated from A431 cells, which contain high levels of activated EGF receptor, displays constitutively enhanced kinase activity.

**MATERIALS AND METHODS**

**Cell Culture and EGF Treatment—**COS-7 and human epidermal carcinoma A431 cells (ATCC) were maintained in high glucose Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (Life Technologies, Inc.), penicillin, and streptomycin at 37 °C in 5% CO₂ (10). For EGF (UBI) (50 ng/ml) studies, cells were placed in serum-free medium for 12 h prior to the treatment.

Expression of KSR in COS-7 and A431 Cells—The plasmids pCDNA3-Flag-KSR and pCDNA3-Flag-KSR (D683A/D700A) were generated as described (10). The N-terminal fragment of KSR (1–541, ΔC-KSR) and C-terminal fragment of KSR (542–875, ΔN-KSR) were subcloned into EcoRI/NotI and EcoRI/XhoI sites of pCDNA3-Flag, respectively, and sequenced.

COS-7 and A431 cells were plated at a density of 1.5 × 10⁵ cells in 150-mm plates (Corning) and grown overnight to approximately 70% confluence. Culture medium was replenished with fresh medium 1 h before transfection using LipofectAmine™ (Life Technologies, Inc.) according to the manufacturer’s instructions. At 48 h post-transfection, cells were placed in serum-free medium for 12 h prior to treatment with EGF. At the indicated times, cells were harvested in Nonidet P-40 buffer (25 mM Tris, pH 7.5, 137 mM NaCl, 10% glycerol, 1% Nonidet P-40, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin/soybean trypsin inhibitor, 5 mM NaVO₄). The homogenate was centrifuged at 10,000 g for 5 min at 4 °C, the supernatant was collected and pre-cleared with Protein A/G-agarose (Amersham Pharmacia Biotech) and protein content was measured using BCA Reagent A (Pierce). Lysates were divided into 500 μg aliquots and stored at −80 °C for subsequent use. KSR expression was determined by Western blot as described (10).

**Immunoprecipitation of KSR—**Flag-tagged proteins were immunoprecipitated from 500 μg of COS-7 and A431 lysates using 60 μl of agarose-conjugated mouse anti-Flag Ab (Sigma) at 4 °C for 2 h. Endogenous KSR was quantitatively immunoprecipitated from 2 μg of A431 lysate at 4 °C for 2 h using 10 μg of rabbit anti-mouse KSR Ab, raised against amino acids 259–281, which overlaps the CA2 domain of mouse KSR (1). Prior to immunoprecipitation, total cell lysate was precleared with 2 μg of normal rabbit IgG for 30 min at 4 °C. Immunoprecipitation with normal rabbit IgG served as control for specificity of the anti-KSR Ab. KSR immune complexes were collected by centrifugation and washed 5 times with Nonidet P-40 lysis buffer containing either 0.15 or 3 M NaCl. The beads were subsequently washed once with reaction buffer (100 mM Tris, pH 7.5, 25 mM β-glycerolphosphate, 5 mM EGTA, 1 mM dithiothreitol, 1 mM NaVO₄) before kinase activity was measured.

**One-stage KSR Activity Assay—**The kinase activity of KSR was determined by *in vitro* reconstitution of the MAPK signaling cascade using MBP phosphorylation as a readout. Amounts of recombinant proteins used in the assay were titrated individually to give the maximal signal-to-noise ratio. The reaction was stopped by the addition of Laemmli buffer. Samples were resolved by 10% SDS-PAGE and autoradiographed. For some studies, EGF or nonactivated MEK1 (KSR-ΔN-MEK1, 500 μg/assay) was used to assess c-Raf-1-dependent activity. We measured KSR kinase activity, the specificity of KSR in mediating activation of the MAPK cascade was confirmed by neutralization of KSR Ab with 10 μg of the KSR peptide against which the Ab was raised for 15 min, prior to performing the immunoprecipitation.

**Two-stage KSR Activity Assay—**To separate KSR from recombinant kinases other than c-Raf-1, a two-stage assay was designed. For these assays, Flag-tagged KSR or endogenous KSR was immunoprecipitated and washed as above. In the first stage, KSR was incubated with 15 μl of reaction mixture containing 60 μM ATP, 7.5 mM MgCl₂ and 8 ng of human c-Raf-1 for 10 min at 30 °C with agitation. Thereafter, the reaction mixture was centrifuged at 14,000 × g for 3 min at 4 °C to pellet the KSR-containing beads, and 10 μl of supernatant containing c-Raf-1 was collected. In the second stage, the activated c-Raf-1 supernatant was added to 20 μl of reaction mixture containing 60 μM ATP, 7.5 mM MgCl₂, 250 ng of unactivated murine GST-MEK1, 1 μg of unactivated murine GST-ERK2/MAPK, 10 μg of MBP or 2 μg of human GST-Elk-1 fusion protein (New England Biolabs, Catalog number 91845), and 30 μCi of [γ-32P]ATP (3000 Ci/mmol) at 30 °C. After a 20-min agitation the reaction was stopped by the addition of 10 μl of Laemmli buffer. Samples were resolved by 10% SDS-PAGE and autoradiographed. For some studies, phosphorylated Elk-1 was visualized by Western blot of 2 μl (containing 100 ng of Elk-1) of the total reaction mixture (40 μl) using a rabbit anti-phospho-Elk-1 (Ser-383) Ab (New England Biolabs, Catalog number 91815). For these studies, [γ-32P]ATP was omitted from the reaction mixture.

**RESULTS**

EGF Treatment Enhances KSR Kinase Activity—To determine whether EGF treatment stimulated KSR activity in mammalian cells, COS-7 cells were transiently transfected with pCDNA3-Flag-KSR using LipofectAmine™. At 48 h post-transfection, cells were placed in serum-free medium and at 60 h post-transfection stimulated with 50 ng/ml EGF. At the times indicated in Fig. 1A, cells were lysed in Nonidet P-40 buffer and KSR was immunoprecipitated from 500 μg of lysate with mouse anti-Flag Ab. Immunoprecipitated KSR was washed three times with Nonidet P-40 lysis buffer containing 150 mM NaCl and once with a buffer containing 50 mM HEPES, pH 7.4, 5.0 mM MgCl₂, and 1 mM dithiothreitol. The kinase activity of KSR was determined by *in vitro* reconstitution of the MAPK signaling cascade using MBP phosphorylation as a readout. For these studies, immunoprecipitated Flag-KSR was combined with recombinant c-Raf-1, MEK1, ERK1, and MBP in an assay buffer containing radiolabeled [γ-32P]ATP as described previously (10). After 30 min, the reaction was stopped, and phosphorylated MBP was resolved by 10% SDS-PAGE and autoradiographed. As shown in Fig. 1A, treatment of COS-7 cells with EGF markedly enhanced the activity of immunoprecipitated KSR to signal MAPK-mediated MBP phosphorylation. (As previously shown and confirmed below in Figs. 1C, 2, and 3, KSR signaling was c-Raf-1-dependent in this assay.) EGF activation of KSR was time-dependent, and the maximal effect was observed at 3 min of EGF treatment. The time course for KSR activation correlated closely with activation of endogenous MBP as measured by anti-phospho-MAPK antibodies (n = 6). A similar time course of KSR activation upon EGF treatment was observed in A431 cells expressing Flag-tagged KSR (not shown). Therefore, the 3-min time point was chosen for EGF treatment throughout these studies.

**Multiple High Salt Washes Remove c-Raf-1, MEK1, and ERK/MAPK from KSR Immune Complexes While Retaining KSR Activity**—To ensure that the kinase activity observed in the *in vitro* reconstitution assay was intrinsic to KSR, we separated KSR from co-immunoprecipitated proteins by high salt washing. For these studies, Flag-tagged KSR immunoprecipitates
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Fig. 1. EGF treatment enhances KSR kinase activity in a high salt-washed immune complex. A, COS-7 cells, transfected with Flag-tagged mouse KSR using LipofectAMINE™, were treated with 50 ng/ml EGF for the indicated times. KSR was immunoprecipitated from 500 μg of lysate for each reaction point, and the kinase activity of KSR was assessed in a one-stage in vitro assay as described under “Materials and Methods.” After 30 min, the reaction was stopped by the addition of Laemmli buffer, and phosphorylated MBP was resolved with 10% SDS-PAGE and autoradiographed. Expression levels of Flag-KSR were similar in all samples as determined by Western blot. Transfection efficiency was estimated by immunofluorescent microscopy at 85–90% by co-transfection with a pTracer-SV40 green fluorescence protein. These data represent 1 of 3 similar experiments. B, Flag-tagged KSR immunoprecipitated from 500 μg of COS-7 lysate was washed sequentially with increasing concentrations of NaCl (0.15, 0.3, 0.5, 0.8, and 1.0 M) in Nonidet P-40 lysis buffer. For example, the KSR immune complex of lane 6 was washed five times sequentially with 0.15, 0.3, 0.5, 0.8, and then 1.0 M NaCl-containing Nonidet P-40 buffer. Proteins in the KSR complex were resolved by 10% SDS-PAGE, transferred onto a polyvinylidene difluoride membrane, and probed with mouse anti-Flag Ab, rabbit anti-human c-Raf-1, or rabbit anti-mouse MEK1 or MAPK Abs. 40 μg of total lysate from COS-7 cells transfected with Flag-KSR was resolved in lane 1. Exposure times for films for KSR, c-Raf-1, MEK1, and ERK2 were 30 s, 10 min, 2 min, and 5 min, respectively. These data represent 1 of 4 similar experiments. C, COS-7 cells, transfected with wild type KSR and KI-KSR, were treated with EGF for 3 min at 60 h post-transfection. KSR immunoprecipitates were washed 5 times with 1.0 M NaCl-containing Nonidet P-40 buffer, and KSR activity was determined by the one-stage in vitro reconstitution assay as in Fig. 1A. In a few studies, trace MEK-1 was detected bound to KSR even after 5 high salt washes, which could be removed by additional washes without alteration of KSR specific activity (not shown). Expression of wild type and KI-KSR was determined by Western blot of 20 μg of total cell lysate using a mouse anti-Flag Ab (inset). These data represent 1 of 5 similar experiments.

(from 500 μg of COS-7 lysate) were washed progressively with increasing concentrations of NaCl from 0.15–1.0 M in Nonidet P-40 lysis buffer (Fig. 1B). For instance, the immunoprecipitate in lane 2 was washed once with 0.15 M NaCl solution whereas the immunoprecipitate in lane 5 was washed four times sequentially with 0.15, 0.30, 0.50, and 0.8 M NaCl solution prior to SDS-PAGE and Western analysis. The lane marked Lysate contained 40 μg of Flag-KSR transfected COS-7 lysate for comparison. When immunoprecipitates were washed with low salt (0.15 M NaCl), endogenous c-Raf-1, MEK1, and ERK/MAPKs were readily detected within the KSR immune complex (Fig. 1B, lane 2). Even after multiple washes with 0.15 M NaCl/Nonidet P-40 buffer these kinases remained bound to KSR (not shown). Multiple high salt washes, however, successfully removed c-Raf-1, MEK1, and MAPKs between 0.3 and 0.8 M NaCl (Fig. 1B, lanes 3–5). After the 1 M NaCl wash, no c-Raf-1, MEK1, or MAPK could be detected in the KSR immune complex (Fig. 1B, lane 6). Prolonged exposure of the films failed to detect these proteins in the KSR immune complex after the 1 M NaCl wash (not shown). Additionally, c-Raf-1, MEK1, and MAPKs were recovered in the supernatants collected after each wash (not shown). Collectively, these results demonstrate that high salt washing was necessary to quantitatively remove c-Raf-1, MEK1, and MAPK from the KSR immune complexes.

The kinase activity of KSR after 1 M NaCl high salt washing was measured in a one-stage in vitro reconstitution assay of MAPK signaling using MBP phosphorylation as the readout.

For these studies, KSR, immunoprecipitated from control or EGF-treated COS-7 cells, was washed five times with 1 M NaCl containing Nonidet P-40 buffer and once with 20 mM Tris-HCl (pH 7.5) and then incubated with a reaction mixture containing recombinant c-Raf-1, MEK1, ERK2/MAPK, and MBP. Under these conditions, greater than 98% of the contaminating proteins detected by silver staining in the original immunoprecipitate were removed by the 1 M NaCl washes whereas over 90% of the KSR remained bound to the beads (Fig. 1B and not shown). The amounts of contaminating proteins used in the assays were titrated individually so that in the absence of KSR, phosphorylation of MBP by these proteins alone was hardly detectable. KSR, when immunoprecipitated from unstimulated COS-7 cells, was unable to enhance MAPK signaling (Fig. 1C, lanes 1–3). However, treatment of COS-7 cells with EGF (50 ng/ml) for 3 min markedly enhanced the capacity of immunoprecipitated KSR to increase MAPK phosphorylation of MBP (Fig. 1C, lane 6). Moreover, signaling through the MAPK cascade in response to KSR isolated from EGF-stimulated cells was c-Raf1-dependent because EGF-activated KSR in the absence of c-Raf1 was unable to enhance MAPK signaling (Fig. 1C, lane 5). c-Raf1-dependent KSR activity was also detected using kinase-inactive MEK1 (K97M-MKK1) as readout for c-Raf1 activity (not shown). Furthermore, nonspecific IgG immunoprecipitates from EGF-treated COS-7 cells overexpressing Flag-tagged KSR were ineffective in initiating signaling through the MAPK cascade (Fig. 1C, lanes 1 and 4). At least 75% of the original KSR activity immunoprecipitated from COS-7 cells and washed with low salt was retained after 1 M NaCl washing (not shown). These studies indicate that high salt washed immunoprecipitated KSR retains the capacity to reconstitute c-Raf1-dependent signaling through the MAPK cascade in vitro. In contrast, kinase-inactive KSR (KI-KSR), when expressed to the same level as wild type KSR (Fig. 1C, inset), did not support EGF-stimulated signaling (Fig. 1C, lanes 7 and 8). These results strongly argue that the kinase activity of KSR is required for its function to enhance signaling through the MAPK cascade in vitro.

Only Full-length Wild Type KSR Can Support MAP Kinase Activation—To further investigate EGF activation of KSR, A431 cells, which express abundant EGF receptors (17), were employed. Flag-tagged KSR, immunoprecipitated from control and EGF-treated A431 cells, was assayed for its capacity to activate MAPK by a two-stage reconstitution assay in vitro. This two-stage assay was designed to separate KSR from re-
were immunoprecipitated from 500 
A431 cells (Fig. 2
)
shown). Again, EGF treatment failed to activate KI-KSR in
yielded comparable sensitivity with greater specificity (not
in vitro
reconstitution of MAPK signaling
activity between COS-7 and A431 cells is explored below.

manifested c-Raf-1-dependent activity in our MAPK reconsti-
1
tion assay (Fig. 2
). Comparison of this alternative method of detection of

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combinant proteins other than c-Raf-1. For these assays, immu-

nomprecipitated KSR was first washed five times with 1 M
NaCl and then incubated with recombinant human c-Raf-1.

Thereafter, the KSR-bound beads were pelleted and the super-
natant containing activated c-Raf-1 was added to the reaction
mixture containing recombinant human MEK1, ERK2, and
Elk-1 as substrate. Elk-1 is a specific ERK substrate, and its
use has the advantage of minimizing nonspecific phosphoryla-
tion. Phosphorylated Elk-1 was visualized by Western blot
analysis using a rabbit anti-phospho-Elk-1 (Ser-383) Ab. Note that prolonged
exposure of films allowed detection of very low levels of Elk-1 phos-
phorylation in the ΔN-KSR mutant, representing <5% of full-length KSR
activity. These data represent 1 of 4 similar experiments. Complete and
even transfer of the resolved proteins to the polyvinylidene difluoride
membrane was monitored by PhastGel Blue R (Life Technologies, Inc.).

B, mutant and wild type KSRs, immunoprecipitated from A431 lysates
in A, were resolved by 10% SDS-PAGE, and expression was determined
by Western blot using a mouse anti-Flag Ab. Phosphorylated
Elk-1, resolved by 10% SDS-PAGE, was visualized by Western blot
was stopped by the addition of Laemmli buffer, and phosphorylated
Elk-1 was resolved by 10% SDS-PAGE, and expression was determined
by Western blot using a mouse anti-Flag Ab (lanes 1–8). Immunopre-
cipitation with normal mouse IgG from lysates transfected with Flag-
tagged wild type KSR served as control (lanes 9 and 10). Similar control
results were obtained with lysates of A431 cells transfected with KSR
mutant constructs (not shown).

As described above, Flag-KSR immunoprecipitated endogenous
KSR to activate MAPK signaling was determined
in vitro.

Endogenous KSR of A431 cells is constitutively active in
the absence of EGF treatment. A, endogenous KSR was quanti-
tatively immunoprecipitated from 2 mg of A431 lysate at 4 °C for 2 h
using 10 μg of rabbit anti-mouse KSR Ab (lane 2). Immunoprecipitation
with normal rabbit IgG served as control for the specificity of anti-KSR
Ab (lane 3). KSR expression was determined by Western blot as de-
scribed under “Materials and Methods.” B, the capacity of immu-
noprecipitated endogenous KSR to activate MAPK signaling was determined
by the one-stage in vitro reconstitution assay as in Fig. 1A. The speci-
icity of KSR in mediating MAPK signaling was assessed by neutral-
ization of the KSR Ab with the KSR peptide from which the Ab was
raised prior to immunoprecipitation (lane 6). C, the capacity of immu-
noprecipitated endogenous KSR to activate MAPK signaling was deter-
mined by the two-stage in vitro reconstitution assay as in Fig. 2A. 1
32P-Phosphorylated Elk-1 was resolved by 10% SDS-PAGE and auto-
radiographed. These data represent 1 of 4 similar experiments.

C-terminal (1–541, ΔC-KSR) deletion mutants in our two-stage
assay. For these studies, equal amounts of full-length KSR,
ΔC-KSR, and ΔN-KSR (and KI-KSR) were immunoprecipitated from untreated or EGF-treated A431 cells (Fig. 2B). In contrast
to full-length KSR, ΔC-KSR and ΔN-KSR failed to support
MAPK activation in vitro (Fig. 2A, lanes 1–4). These results
demonstrate that full-length kinase-active KSR is the only form of KSR capable of signaling c-Raf-1-dependent MAPK
activation in an in vitro reconstitution assay. Similar results
were obtained using COS-7 cells (not shown).

Endogenous KSR of A431 Cells Is Constitutively Active in
the Absence of EGF Treatment. As described above, Flag-KSR
immunoprecipitated from A431 cells was active in the absence
of EGF treatment (Fig. 2A, lanes 9) while it was inactive when
immunoprecipitated from resting COS-7 cells (Fig. 1C, lane 3).
This suggests that endogenous KSR from A431 cells, which
signal constitutively through the EGF receptor, might also be
active. To address this issue, we raised an anti-mouse KSR Ab
in rabbits against amino acids 259–281 of the CA2 domain of
mouse KSR. This region is identical to the corresponding region
of human KSR (1). As shown by Western blot (Fig. 3A), this Ab
quantitatively immunoprecipitated endogenous KSR from 2
mg of A431 lysate (lane 2). 40 μg of total lysate from A431 cells transfected with Flag-tagged KSR was used for comparison (Fig. 3A, lane 1). Normal rabbit IgG failed to immunoprecipitate endogenous KSR from A431 cells, indicating high specificity of the anti-mouse KSR Ab (Fig. 3A, lane 3). For these studies, immunoprecipitates were washed 5 times with 1.0 M NaCl without significant depletion of endogenous KSR bound to the beads (not shown).

Using this Ab, the capacity of endogenous KSR from A431 cells to activate MAPK signaling was determined by both the one-stage (Fig. 3B) and two-stage (Fig. 3C) in vitro reconstitution assays. High salt washed immunoprecipitates of endogenous KSR from resting A431 cells, like overexpressed KSR, reconstituted MAPK signaling in a c-Raf-1-dependent fashion. The level of endogenous KSR kinase activity was quite high, as phosphorylated MBP was readily detectable after only 20 min of autoradiography at −80 °C (Fig. 3B, lane 4). However, prolonged exposure of films up to 12 h allowed visualization of very low levels of MBP phosphorylation in experimental controls (Fig. 3B, lanes 1–3, 5, and 6 and not shown). Direct activation of ERK2/MAPK or MBP by KSR was not observed (Fig. 3B, lanes 2 and 3). Neutralization of the KSR Ab by preincubation with the peptide against which the Ab was raised (Fig. 3B, lane 6), prior to immunoprecipitation, provided evidence that reconstitution of MAPK signaling in vitro by endogenous KSR was specific. To confirm these observations, the capacity of immunoprecipitated endogenous KSR to activate MAPK signaling was also determined by the two-stage assay using 32P incorporation into recombinant GST-Elk-1 as the readout. Similar to what was observed in the one-stage assay (Fig. 3B), KSR-mediated phosphorylation of GST-Elk-1 by ERK2/MAPK was c-Raf-1-dependent and was readily detected after only 15 min of autoradiography (Fig. 3C). Collectively, these results demonstrated that endogenous KSR of A431 cells is constitutively active.

**DISCUSSION**

The present investigations use an improved KSR kinase assay to demonstrate c-Raf-1-dependent reconstitution of the MAPK signaling cascade in vitro. KSR activity could not be reconstituted in assays lacking recombinant c-Raf-1, and KSR conferred MEK1 activation capabilities onto c-Raf-1 in a two-stage assay. Only full-length kinase-active KSR appeared capable of signaling c-Raf-1-dependent activity as C- and N-terminal deletion mutants were without effect. Further, functional integrity of the KSR kinase domain appeared necessary because substitution of conserved aspartates involved in phospho-transfer with alanines (KI-KSR) abrogated MAPK signaling. However, the recognition of the EGF pathway as a Ras-effector molecule.

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