KSR-1 Binds to G-protein βγ Subunits and Inhibits βγ-induced Mitogen-activated Protein Kinase Activation*

(Received for publication, October 20, 1998, and in revised form, December 23, 1998)

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The protein kinase KSR-1 is a recently identified participant in the Ras signaling pathway. The subcellular localization of KSR-1 is variable. In serum-deprived cultured cells, KSR-1 is primarily found in the cytoplasm; in serum-stimulated cells, a significant portion of KSR-1 is found at the plasma membrane. To identify the mechanism that mediates KSR-1 translocation, we performed a yeast two-hybrid screen. Three clones that interacted with KSR-1 were found to encode the full-length γ₁₀ subunit of heterotrimeric G-proteins. KSR-1 also interacted with γ₂ and γ₃ in a two-hybrid assay. Deletion analysis demonstrated that the isolated CA3 domain of KSR-1, which contains a cysteine-rich zinc finger-like domain, interacted with γ subunits. Coimmunoprecipitation experiments demonstrated that KSR-1 bound to β₁γ subunits when all three were transfected into cultured cells. Lysophosphatidic acid treatment of cells induced KSR-1 translocation to the plasma membrane from the cytoplasm that was blocked by administration of pertussis toxin but not by dominant-negative Ras. Finally, transfection of wild-type KSR-1 inhibited β₁γ-induced mitogen-activated protein kinase activation in cultured cells. These results demonstrate that KSR-1 translocation to the plasma membrane is mediated, at least in part, by an interaction with βγ and that this interaction may modulate mitogen-activated protein kinase signaling.

The Ras signaling pathway affects many aspects of cell physiology, including cell growth, proliferation, movement, and differentiation (1). Recently, KSR-11 was identified as a component of the Ras signaling cascade by genetic screens in Drosophila melanogaster and Caenorhabditis elegans (2–4). Inactivating mutations in the ksr-1 gene blocked the phenotypic effects of activated Ras in these animals, suggesting that KSR-1 is a positive regulator of Ras-mediated signaling. Genetic epistasis experiments in Drosophila demonstrated that KSR-1 acts downstream of Ras but upstream of or parallel to Raf (4).

Mammalian forms of KSR-1 have been identified on the basis of sequence homology (4), but the role of mammalian KSR-1 in Ras-mediated signaling is controversial. Overexpression of KSR-1 in Xenopus oocytes was found by two groups (including ours) to weakly promote MAP kinase activation (5–7). In one study (6), overexpression of KSR-1 in cultured mammalian cells was found to promote MAP kinase activation; it was shown to inhibit Ras-mediated signaling at the level of MEK activation in several studies (8–10), and it was found to inhibit Ras-mediated signaling at the level of transcription factor (Elk-1) activation (11). In the absence of loss-of-function studies in mammalian cells, the definitive role of KSR-1 in Ras-mediated signaling remains unclear.

Both invertebrate and mammalian forms of KSR-1 consist of a putative amino-terminal regulatory portion and a carboxyterminal serine/threonine kinase domain. Five functional domains of KSR-1 have been identified, including a unique amino-terminal CA1 domain, a proline-rich CA2 domain, a cysteine-rich zinc finger-like CA3 domain, a serine/threonine rich CA4 domain, and the amino-terminal protein kinase CA5 domain (4). KSR-1 is most homologous to Raf-1 kinase, but there is no evidence that KSR-1 can bind to Ras or phosphorylate MEK. Indeed, the in vivo substrate(s) of the kinase domain of KSR-1 is unknown (7, 11).

One model of KSR-1 action purports that it is a molecular scaffold that behaves like the budding yeast protein ste5, functionally linking the protein kinases ste11, ste7, and fus3/kss1 (6). Indeed, KSR-1 has been shown to interact with 14–3-3 protein, Raf-1, MEK, and MAP kinase in coimmunoprecipitation experiments and yeast two-hybrid assays (5–10). It is not clear, however, whether KSR-1 links these associated proteins to promote signal transduction. Mutational analysis of KSR-1 has revealed that separable domains bind to distinct signaling proteins. For example, in vitro binding assays have demonstrated that the CA4 domain of KSR-1 interacts with MAP kinase (12). Furthermore, yeast two-hybrid assays have shown that the CA5 domain of KSR-1 binds to MEK (8, 9).

The subcellular localization of KSR-1 is dependent on the activation state of cells. We previously demonstrated that KSR-1 is a cytoplasmic protein in serum-starved cells, but that KSR-1 translocates to the plasma membrane after stimulation with serum (5). The time course of this translocation is similar to that observed for Raf-1 kinase, which binds to activated Ras at the plasma membrane. Work by Michaud et al. (7) has established that the cysteine-rich CA3 domain of KSR-1 is essential for translocation to the plasma membrane. One explanation for this observation is that KSR-1 accompanies Raf-1 to the plasma membrane, but inactive Raf-1 does not bind to KSR-1 (5). Another possibility is that KSR-1 directly binds to a constitutively membrane-bound target after serum stimulation. To further explore this possibility, we performed a yeast two-hybrid screen using KSR-1 as bait.

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** Supported by grants from the Barnes-Jewish Hospital Foundation, the National Institutes of Health, and the American Heart Association.

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‡ The abbreviations used are: KSR-1, kinase suppressor of Ras; mKSR-1, murine KSR-1; FCS, fetal calf serum; LFA, lysophosphatic acid; PTX, pertussis toxin; PAGE, polyacrylamide gel electrophoresis; GST, glutathione S-transferase; X-gal, 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside; ERK, extracellular signal-regulated kinase; MAP, mitogen-activated protein; MEK, MAP kinase or extracellular signal-regulated kinase.
Experimental Procedures

Yeast Two-hybrid Screen—A cDNA encoding the CA2 through CA5 domains (amino acids 195 to 873) of murine KSR-1 (mKSR-1) was inserted into the vector pAS1-CYH (gift of Stephen Elledge, Baylor University, Houston, Texas) as an in-frame fusion with the transactivation domain of GAL4 (pAS1/CA2–5) as described previously (12). A human HeLa cell cDNA two-hybrid library (gift of David Beach, Cold Spring Harbor Laboratory, New York) was screened and pAS1/CA2–5 was used as the bait. A yeast strain (Y190) was cotransfected with pAS1/CA2–5 and the HeLa cell library and yeast were plated onto medium lacking leucine and tryptophan. Colony lifts were analyzed for β-galactosidase activity by use of X-gal, and colonies that exhibited detectable activity were scored positive (+).

Mammalian Expression Constructs—The full-length mKSR-1 cDNA (gift of Marc Therrien and Gerald Rubin, University of California, Berkeley) was subcloned into the pTarget (Promega) mammalian expression vector. The human wild-type β2, γ2, and γ10 cDNAs were subcloned into the mammalian expression vector pCB6+. A 5′-FLAG-tagged version of mKSR-1 was obtained by use of polymerase chain reaction and was subcloned directly into pTarget (Promega) and sequenced. The N17 Ras cDNA was a gift from Dwight Towler (Washington University, St. Louis, MO).

Antibodies—The rabbit polyclonal anti-β2 subunit antibody (BN-1) has been previously described (15). The rabbit polyclonal anti-pan-β subunit, the goat polyclonal anti-KSR-1, and the rabbit polyclonal anti-ERK1 antibodies were obtained from Santa Cruz Biotechnology. The alkaline phosphatase-conjugated rabbit anti-goat IgG secondary antibody was obtained from Zymed Laboratories Inc. The alkaline phosphatase-conjugated goat anti-rabbit IgG secondary antibody was obtained from Santa Cruz Biotechnology.

In Vitro Association Experiments—cDNAs encoding the CA1 and CA2 (amino acids 1–311), CA3 (amino acids 312–392), CA4 (amino acids 384–519), or CA5 (amino acids 520–873) domains of mKSR-1 were inserted in-frame into pGEX-4T (Amersham Pharmacia Biotech) that contained the coding sequence for glutathione S-transferase (GST). GST fusion proteins were produced in Escherichia coli and were immobilized on glutathione beads (Sigma). NIH/3T3 fibroblasts were lysed in Nonidet P-40 lysis buffer (0.5% Nonidet P-40, 137 mM NaCl, 50 mM NaF, 10 mM Tris, pH 7.5, 3 mM dithiothreitol, 6 mM MgCl2, 2 mM phenylmethylsulfonyl fluoride, 0.2 units/ml aprotinin, 25 μM leupeptin). Lysates were cleared by low speed centrifugation (12,000 × g for 5 min) and then added to immobilized GST fusion protein. Protein lysate derived from approximately 106 cells was added to 0.5 μg of immobilized GST fusion protein. Beads were washed three times with lysis buffer, and adherent proteins were boiled in gel sample buffer. Proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and electrothermally transferred to nitrocellulose filters. Filters were blocked in 3% nonfat dry milk and 3% bovine serum albumin in TBS/T (10 mM Tris, pH 7.5, 150 mM NaCl, 0.1% Tween 20). After incubation in primary antibody, bound antibody was visualized with alkaline-phosphatase-conjugated secondary antibody and color-developing agents (Promega).

Cell Culture and Transfections—COS7 cells were maintained in Dulbecco’s modified Eagle’s medium with 10% fetal calf serum. Cells were split 24 h before transfection with the indicated cDNAs. All transfections were carried out with the use of LipofectAMINE Plus reagent (Life Technologies, Inc.) in serum-free medium (OptiMEM, Life Technologies, Inc.) in serum-free medium (OptiMEM, Life Technologies, Inc.).

Coimmunoprecipitation Assays—For triple transfection experiments, 1–3 × 106 COS7 cells in 100-mm culture plates were transfected with the cDNAs encoding β2 (2.5 μg), γ2 (2.5 μg), and mKSR-1 (2.5 μg). Two days later, transfected COS7 cells were lysed in Nonidet P-40 lysis buffer and cleared by low speed centrifugation (50). Goat anti-KSR-1 antibody (Santa Cruz Biotechnology, 1:100 dilution) or goat anti-rabbit

![FIG. 1. The CA3 domain of mKSR-1 interacts with G-protein γ subunits by two-hybrid assay.](Image)
Association of mKSR-1 with \( \gamma \) subunits in vitro and in vivo.

A. in vitro association of the CA3 domain of mKSR-1 with \( \beta \gamma \) subunits. Bacterially expressed GST fusion proteins were immobilized on glutathione beads, and samples were incubated with NIH/3T3 cell lysates. The beads were washed and adherent proteins were analyzed by immunoblotting by use of an anti-pan-\( \beta \) subunit antibody (Santa Cruz Biotechnology). The fusion proteins contained protein fragments corresponding to GST alone (GST), the CA1 and CA2 domains (GST-CA1CA2), the CA3 domain (GST-CA3), the CA4 domain (GST-CA4), and the CA5 domain (GST-CA5) of mKSR-1.

B. in vivo association of mKSR-1 with \( \beta \gamma \) subunits in transfected cells. COS7 cells were triple transfected with mammalian expression vectors encoding wild-type mKSR-1, human \( \beta_{1} \gamma_{1} \), and human \( \gamma_{1} \). Anti-\( \beta \)-Ras or control (rabbit IgG) immunoprecipitates were analyzed by immunoblotting by use of an anti-\( \beta_{1} \) subunit antibody (BN-1). Protein lysates derived from \( 5 \times 10^{5} \) cells were used for each immunoprecipitation. This immunoblot is representative of the results of three separate experiments.

C. in vivo association of mKSR-1 with \( \beta \gamma \) subunits in untransfected cells. Untransfected COS7 cells were cultured in the absence of serum for 24 h and then some were stimulated with 10% bovine serum for 10 min. Anti-\( \beta \)-Ras or control (rabbit IgG) immunoprecipitates were analyzed by immunoblotting by use of an anti-pan-\( \beta \)-subunit antibody. Protein lysates derived from 10^5 cells were used for each immunoprecipitation. This immunoblot is representative of the results of four separate experiments.

FIG. 3. Translocation of mKSR-1 from the cytosolic fraction to the membrane fraction of cell lysates. A, mKSR-1 translocates to the membrane fraction in response to LPA stimulation. COS7 cells that were transfected with wild-type mKSR-1 were cultured in the absence of serum (Unstimulated) and then were stimulated with LPA or 10% FCS. Some cells were preincubated with PTX before LPA stimulation (LPA/PTX). Detergent-free cell lysates were preincubated, and then separated by high speed centrifugation (100,000 \( \times g \) for 1 h). Supernatants (S100) were reserved, and pellets (P100) were resuspended in an equal volume of buffer with added 1% Triton X-100. Fractions were analyzed by immunoblotting by use of an anti-KSR-1 or an anti-pan-\( \beta \) subunit polyclonal antibody (Santa Cruz Biotechnology). B, mKSR-1 translocation in response to LPA is blocked by PTX, but not by dominant-negative (N17) Ras. COS7 cells were transfected with wild-type mKSR-1 or with mKSR-1 and N17 Ras. Cells were cultured in the absence of serum, and some were stimulated with LPA or 10% FCS, whereas others were preincubated with PTX before LPA stimulation. Cell lysates were separated and analyzed as described in A. Three subcellular fractionation experiment were downloaded to a computer and analyzed by densitometry with NIH Image software. The data are presented as the mean percent of mKSR-1 in the membrane fraction (membrane fraction + cytosolic fraction = 100%) ± S.E.
fluoride, and 1 mM Na3VO4. Immunoprecipitates were then resus-
pended. Parallel samples were analyzed by immunoblotting with an
anti-ERK antibody, 20
m
P-40 lysis buffer to which NaCl had been added (1M final concentration)
Biotechnology) was added to each reaction to immobilize antibody-
were double-transfected with mammalian expression vectors encoding
a substrate. COS7 cell lysates were obtained from control untransfected
lysates were analyzed by immunoblotting with an anti-ERK subunit polyclonal antibody (BN-1).

RESULTS

We performed a yeast two-hybrid screen using a cDNA that
coded the CA2 through CA5 domains of mKSR-1 (CA2–5) as
bait with a HeLa cell two-hybrid library. Three positive clones
were found to encode the γ10 subunit of heterotrimeric G-
proteins (18); the subunit did not interact with the protein
kinase Mas or with nuclear lamin on two-hybrid assay. In
subsequent experiments with the two-hybrid assay to deter-
mine whether the interaction between CA2–5 and γ subunits
was specific for γ10, we found that γ2 and γ3 also bound to
CA2–5 (Fig. 1). Several additional deletion mutant forms of
mKSR-1 were then generated to identify the portion of mKSR-1
that was interacting with these subunits (Fig. 1A). The cys-
teine-rich zinc finger-like CA3 domain of mKSR-1 interacted
with γ3, γ2, and γ10 in the two-hybrid assay (Fig. 1B), whereas
neither the proline-rich CA2 domain nor the protein kinase
CA5 domain of mKSR-1 interacted with any of the subunits. A
construct that contained the CA4 domain was found to be
transcriptionally active on its own in yeast.

In living cells, β and γ subunits are obligatorily bound to
each other (19). We therefore investigated the ability of the
isolated CA3 domain of mKSR-1 to bind to βγ subunits derived
from cultured cell protein lysates. Recombinant GST fusion
proteins that contained the CA1 and CA2 domains, the CA3
domain, the CA4 domain, or the CA5 domain of mKSR-1 were
immobilized on glutathione beads and were incubated with
NIH/3T3 cell protein lysates. Adherent proteins were analyzed
by anti-β subunit immunoblotting, because the larger β sub-
unit is more readily detectable on immunoblots than the γ
subunit, and revealed that βγ subunits bound to immobilized
GST-CA3, but not to GST-CA1CA2, GST-CA4, or GST-CA5
(Fig. 2A). We have previously demonstrated that GST-CA4
specifically interacts with MAP kinase (12), and that GST-CA5
binds to MEK.

The ability of KSR-1 to interact with βγ subunits in vivo was
examined in communoprecipitation experiments. COS7 cells
were triple transfected with β1, γ2, and mKSR-1. Anti-KSR
immunoprecipitates obtained from transfected cell protein ly-
sates were analyzed by anti-β subunit immunoblotting, and
this showed that β1γ2 and KSR-1 form a complex in vivo (Fig.
2B). The efficiency of this interaction was determined in three
separate experiments by densitometric analysis of anti-KSR
and anti-β subunit immunoblots: 57% ± 15% (S.E.) of β1γ2
bound to KSR-1.

The ability of KSR-1 to interact with βγ subunits in trans-
fected cells was also investigated. Untransfected COS7 cells
were cultured in the absence of serum for 24 h and then some
cells were stimulated with 10% fetal calf serum for 10 min.
Anti-KSR immunoprecipitates obtained from untransfected cell
protein lysates were analyzed by anti-pan-β subunit immu-
oblotting and this showed that βγ subunits and KSR-1 form a complex
in serum-stimulated but not in serum-starved cells (Fig. 2C).

The ability of βγ subunits to form a complex with KSR-1 in vivo suggested that the liberation of free βγ subunits on G-
protein activation could cause KSR-1 to translocate to the
plasma membrane. We have previously demonstrated that se-
rum stimulation of cultured NIH/3T3 cells results in a redis-
tribution of a significant proportion of KSR-1 from the cytoplas-
mic fraction of cell lysates to the plasma membrane fraction (5).
Because LPA is an important component of serum that binds to
G-coupled receptors, we examined whether LPA could also
induce this translocation of KSR-1 (20). Cultured COS7 cells
were treated with LPA, and the subcellular localization of
KSR-1 was examined by differential centrifugation followed
by immunoblotting. LPA treatment of cells resulted in robust re-
distribution of KSR-1 to the membrane fraction of cell lysates
that was blocked by pretreatment with PTX, which specifically
inhibits G (Fig. 3). Because LPA stimulates G-coupled recep-
tors that can activate Ras (20), we wished to evaluate whether
KSR-1 translocation was dependent on Ras activation. Trans-
fection of cells with dominant-negative (N17) Ras did not in-
hibit LPA-induced redistribution (Fig. 3B) (21).

To evaluate the biological significance of the interaction be-
 tween βγ subunits and KSR-1, we evaluated MAP kinase activity
in transfected cells. Cotransfection of cultured cells with β and γ
subunits has previously been shown to result in MAP kinase
activation in the absence of serum stimulation (22). It has been

\footnote{Heming Xing and Anthony J. Muslin, unpublished observations.}
demonstrated that free βγ subunits interact with and activate phosphatidylinositol 3-kinase, and this interaction is thought to eventually lead to activation of Ras and MAP kinase (23). In this study the additional translocation of cultured cells with full-length mKSR-1 markedly inhibited βγ-induced MAP kinase activation without affecting β1 subunit protein levels (Fig. 4).

**DISCUSSION**

The subcellular localization of many signaling proteins is highly regulated and is often an important determinant of their activity. Localization is thought to influence activity by increasing the proximity of an enzyme to activating molecules or to substrates. For example, the protein kinase Raf-1 must translocate to the plasma membrane to be fully activated (17, 18, 24). The protein kinase KSR-1 also translocates from the cytosol to the plasma membrane, but the importance of this event in regulating the activity of KSR-1 has not been determined. In the experiments described here, we evaluated the mechanism of the translocation of KSR-1 from the cytosol to the plasma membrane. By use of the yeast two-hybrid assay we confirmed that KSR-1 can interact with the γ2, γ5, and γ10 subunits of heterotrimeric G-proteins. These G-protein subunits are lipid modified and have been shown to be constitutively plasma membrane-bound (19). We also demonstrated that KSR-1 binds to βγ subunits in cultured mammalian cells, and that a ligand that liberates βγ subunits, LPA, can stimulate the translocation of KSR-1 to the plasma membrane. These findings confirm that βγ subunits can mediate the translocation of KSR-1 to the plasma membrane.

One interesting aspect of the interaction between γ subunits and KSR-1 is that βγ effectors usually bind directly to the larger β subunit (25–27). The surface of the βγ dimer that interacts with effectors has been examined by x-ray crystallography, demonstrating that there are several distinct areas that interact with effectors (28–30), particularly the amino-terminal coiled-coil domain of β subunit, which is immediately adjacent to the amino-terminal domain of the γ subunit that also forms a coiled-coil (31–33). The amino termini of both subunits form a continuous surface that could theoretically interact with effectors although it remains to be determined whether this is the site of interaction with KSR-1.

We demonstrated by two-hybrid assay that the CA3 domain of KSR-1 can bind to γ2, γ5, and γ10: This domain is highly homologous to the cysteine-rich domains of Raf-1, A-Raf, protein kinase Cα, and citron kinase, and is less homologous to the cysteine-rich domains of diacylglycerol kinase, the racGAP N-terminal domain, MEK to bind to CA5 (8, 9), and MAP kinase to bind to CA4 (12) supports the hypothesis that mKSR-1 is a scaffolding protein. In budding yeast, ste5 links βγ subunits (ste4, ste18) to the MAP kinase cascade proteins ste11, ste7, and fus3/kss1 and promotes their activation (35, 36). In marked contrast to findings with ste5, we found that overexpression of mKSR-1 inhibits βγ-induced MAP kinase activation. This discrepancy suggests that mKSR-1 has a unique physiologic role in the regulation of MAP kinase signaling. Our findings complement recent work by other investigators demonstrating that overexpression of mKSR-1 in cultured mammalian cells inhibits serum- and ligand-induced MAP kinase activation (8–10).

**Acknowledgments**—We thank Maurine Linder and Ken Blumer for technical advice and helpful comments. We thank David Beach, Steve Elledge, Wendy Fantl, Gerald Rubin, Marc Thirrien, and Dwight Towler for reagents.

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