The protein kinase KSR interacts with 14-3-3 protein and Raf

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Background: KSR (kinase suppressor of Ras) is a recently identified putative protein kinase that positively mediates the Ras signaling pathway in the invertebrates *Caenorhabditis elegans* and *Drosophila melanogaster*. The function of vertebrate KSR is not well characterized biochemically or biologically.

Results: We examined the physiological role of KSR in vertebrate signal transduction using *Xenopus laevis* oocytes. Overexpression of KSR, in combination with overexpression of the intracellular dimeric protein 14-3-3, induced *Xenopus* oocyte meiotic maturation and cdc2 kinase activation; the effect of KSR and 14-3-3 on oocyte maturation was blocked by co-expression of dominant-negative Raf-1. We noted that KSR contains multiple potential binding sites for 14-3-3, and we used the yeast two-hybrid system and co-immunoprecipitation experiments to show that KSR can bind to 14-3-3. Furthermore, we demonstrated that KSR can form a complex with Raf kinase both *in vitro* and in cultured cells. Cell fractionation studies revealed that KSR formed a complex with 14-3-3 in both the membrane and cytoplasmic fractions of cell lysates; however, KSR only formed a complex with Raf-1 in the membrane fraction.

Conclusions: Our findings suggest that KSR, 14-3-3 and Raf form an oligomeric signaling complex and that KSR positively regulates the Ras signaling pathway in vertebrate organisms.

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Background

The Ras signaling pathway plays a critical role in the regulation of cell differentiation, movement and proliferation [1]. Activated, GTP-bound Ras binds to Raf, and this interaction promotes Raf activation. Some evidence suggests that a critical function of Ras is to localize Raf to the plasma membrane where Raf may be post-translationally modified and activated [2,3]. Although the activation of Raf is known to be critical for Ras-mediated signaling, the proteins that function in conjunction with Ras to activate Raf have not been definitively identified. The activation of Raf *in vivo* may require its phosphorylation at various serine, threonine and tyrosine residues; there is some evidence for the involvement of each of several specific Raf residues: Ser259, Ser499, Ser621, Thr269, Tyr340 and Tyr341 [4–8]. However, the relative contribution to Raf activation *in vivo* of phosphorylation at each of these residues has not been clearly established.

Raf appears to be part of a high molecular weight complex within cells [9], and this complex is likely to include members of two groups of proteins that are constitutively bound to Raf in many cell types: heat shock proteins and 14-3-3 proteins [9–14]. Raf activation appears to be potentiated by members of the 14-3-3 protein family — a highly conserved group of dimeric proteins known generically as 14-3-3 [10–14]. Although the mechanism of action of 14-3-3 is poorly understood, some evidence suggests that binding to 14-3-3 protects Raf from dephosphorylation [4,15]. Alternatively, 14-3-3 may function either as a linker, by assembling Raf and other signaling proteins into a complex [16], or as a chaperone, by stabilizing Raf in a conformation which is accessible for activation [15]. Several important signaling proteins in cells interact with 14-3-3 [11], which binds to proteins containing the serine-phosphorylated motif Arg–Ser–X–pSer–X–Pro (in which X is any amino acid and pSer is phosphoserine) [15]; 14-3-3 may also bind to proteins that contain cysteine-rich zinc-finger domains [6].

The KSR (kinase suppressor of Ras) family of proteins was recently discovered and shown to be involved in Ras signaling [17–19]. Genetic analyses of the *Caenorhabditis elegans ksr-1* gene and the *Drosophila melanogaster ksr* gene suggest that the products of these genes positively mediate Ras signaling and function downstream of, or parallel to, Ras [17–19]. Genes homologous to the invertebrate *ksr* genes have been identified in mice and humans, but the function of these genes is not well defined [19]. The proteins predicted to be encoded by the *ksr* genes contain a highly conserved protein kinase domain, a cysteine-rich zinc-finger-like domain, and two amino-acid sequences related to the 14-3-3-binding motif Arg–Ser–X–Ser–X–Pro [15]. KSR proteins appear to define a distinct group of protein kinases, which most closely resemble members of the Raf family.

In order to determine the role of KSR in vertebrate signal transduction, we examined the ability of KSR to potentiate meiotic maturation in *Xenopus laevis* oocytes — a process which is believed to be mediated by the Ras signaling pathway, involving the activation of Mitogen-activated protein (MAP) kinase and culminating in germinal vesicle breakdown (GVBD) and the activation of meiotic maturation markers such as cdc2 kinase activity [20]. We also investigated whether KSR can bind to 14-3-3 and Raf. Our results suggest that KSR potentiates the stimulation of Raf-mediated signaling by 14-3-3, and that KSR is able to form a complex with both Raf and 14-3-3.

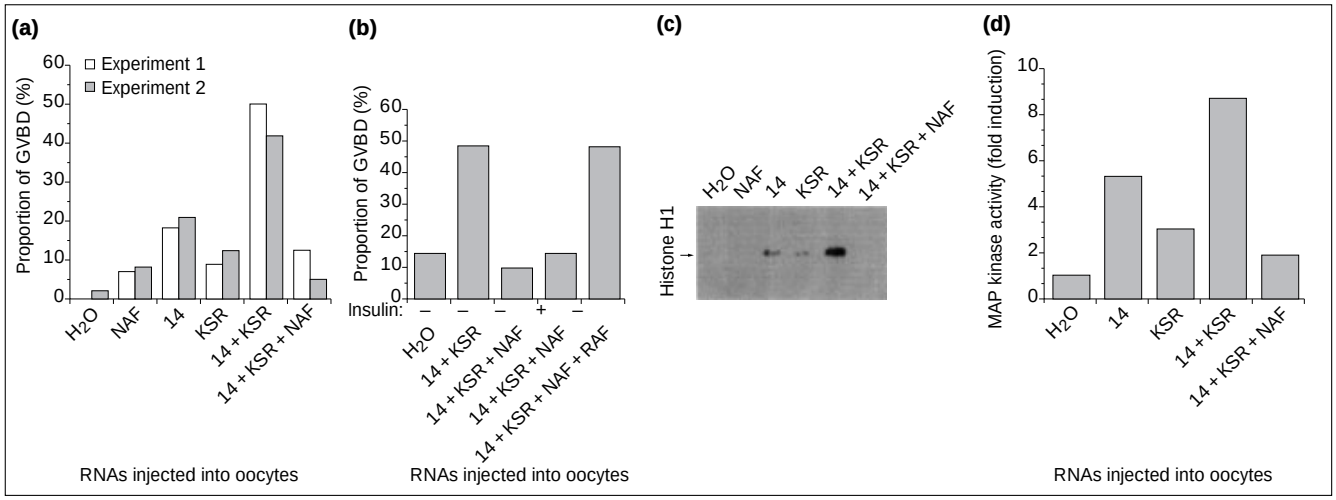
Results

KSR cooperates with 14-3-3 to promote *Xenopus* oocyte maturation

Genetic epistatic analysis in *Drosophila* suggests that *ksr* functions upstream of Raf to positively mediate Ras signaling [19]. Although homologous vertebrate *ksr* genes have been cloned on the basis of sequence similarity [19], the function of vertebrate KSR proteins in signal transduction is not well defined. We first tested the ability of murine KSR (muKSR) to activate the Ras signaling pathway using a well-defined bioassay — meiotic maturation of *Xenopus* oocytes [20]. The muKSR cDNA was used to generate RNA by transcription *in vitro*; RNA was injected into immature oocytes and GVBD and cdc2 kinase activity were assessed. Injection of 10–20 pg muKSR RNA per oocyte did not induce significantly more GVBD or activation of cdc2 kinase than did injection of water as a control (Fig. 1a,c). Injection of muKSR RNA resulted in GVBD in 9–12% of oocytes compared to 6–8% for control oocytes (Fig. 1a); doubling the dosage of muKSR RNA did not increase the fraction of oocytes that displayed GVBD (data not shown).

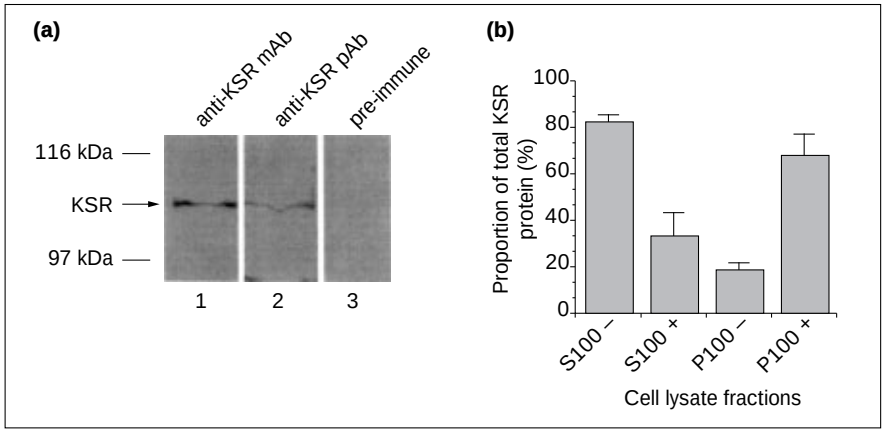
We next tested the ability of KSR to promote maturation in oocytes that were stimulated by expression of one form of human 14-3-3, 14-3-3 ζ. Injection of 10–20 pg of 14-3-3 ζ RNA per oocyte induced GVBD in 18–21% of oocytes (Fig. 1a), consistent with previously reported results [12]. Co-injection of 14-3-3 ζ and muKSR RNAs (20–40 pg RNA in total) resulted in GVBD in 42–50% of oocytes, as determined in eight independent experiments (Fig. 1a), as well as a marked increase in the rate at which GVBD was detected (data not shown). In addition to an

Figure 1



(a) KSR stimulates *Xenopus* oocyte maturation when co-expressed with 14-3-3. Immature *Xenopus* oocytes were injected with RNAs encoding muKSR (KSR), human 14-3-3 ζ (14), and dominant negative Raf (NAF) in various combinations. Groups of 40–50 oocytes were scored for GVBD after 24 h by assessing the appearance of a broad white spot at the animal pole of the oocyte. Two representative sets of data (out of 8) are shown. As a control, water (H_2O) was injected instead of RNA. (b) Wild-type Raf antagonizes the effect of NAF on KSR-induced oocyte maturation. Immature *Xenopus laevis* oocytes were injected with RNAs encoding muKSR (KSR), human 14-3-3 ζ (14), wild type Raf (RAF), and dominant negative Raf (NAF) in various combinations. Some oocytes were stimulated with 8.25 μg ml^{-1} insulin. Groups of 40–50 oocytes were scored for GVBD after 24 h. One representative set of data (out of 3) is shown. As a control, water (H_2O) was injected instead of RNA. (c) Cdc2 kinase is activated by 14-3-3 and KSR in oocytes. Using $p13^{suc}$ beads, cdc2 kinase was purified from *Xenopus* oocytes that had been injected with the various combinations of RNAs used in (a). Histone H1 was added to immobilized cdc2 in the presence of [^{32}P]ATP, and the radiolabeled proteins were analyzed by SDS–PAGE followed by autoradiography. These data are representative of three separate experiments. (d) MAP kinase is activated by 14-3-3 and KSR in oocytes. MAP kinase was purified from injected *Xenopus* oocytes, labeled as in (a), using agarose-conjugated anti-MAP-kinase antisera. Myelin basic protein was added to immobilized MAP kinase in the presence of [^{32}P]ATP, radiolabeled proteins were separated by SDS–PAGE, and the bands corresponding to myelin basic protein were isolated and analyzed by scintigraphy. Each column is the average of two experiments. Data are expressed as the fold induction of MAP kinase activity over control (the activity from water-injected oocytes).

Figure 2



(a) Immunoblot of murine fibroblast protein lysates using anti-KSR antisera. Lysates from subconfluent NIH 3T3 cells were separated by SDS–PAGE and analyzed by immunoblotting using a murine monoclonal anti-KSR antibody (lane 1), a goat polyclonal anti-KSR antibody (lane 2), or rabbit pre-immune serum (lane 3). The migration of protein size markers and bands likely to correspond to KSR are indicated. A second goat polyclonal anti-KSR antibody (Santa Cruz Biotech) also bound to a 105 kDa band (data not shown). (b) Subcellular localization of KSR. Quiescent NIH 3T3 cells (cells incubated for 24 h in medium containing 0.1% fetal calf serum; labeled '–') and serum-stimulated NIH 3T3 cells (quiescent cells stimulated with medium containing 10% fetal calf serum for 10 min; labeled '+') were lysed in detergent-free lysis buffer and separated by high-speed centrifugation. Cytosolic (S100) and membrane (P100) fractions were analyzed by immunoblotting using a goat polyclonal anti-KSR antibody. Bands corresponding to KSR were quantified by densitometry using NIH Image. Each column is the mean densitometric analysis of three independent experiments (± SEM).

increase in GVBD, co-injected oocytes displayed a significant increase in cdc2 kinase activity (Fig. 1c) and MAP kinase activity (Fig. 1d).

A dominant negative form of Raf (NAF) that contains a point mutation at the ATP binding site (Lys375→Met) was also used for oocyte injection experiments; co-injection of three RNAs — 14-3-3 ζ, muKSR and NAF (30–60 pg RNA in total) — reduced GVBD, cdc2 kinase activity, and MAP kinase activity to basal levels (Fig. 1a,c,d). Insulin treatment of oocytes results in Ras and Raf activation and leads to oocyte maturation [21,22]. Treatment of oocytes injected with 14-3-3 ζ, muKSR and NAF RNAs with insulin did not potentiate GVBD; however, co-injection of a fourth RNA, wild-type Raf RNA, (40–80 pg RNA in total) resulted in marked induction of GVBD (Fig. 1b), suggesting that the effect of NAF was specific to Raf. Taken together, these results demonstrate that KSR and 14-3-3 cooperate to promote GVBD in a Raf-dependent manner.

Subcellular localization of KSR

The results of the oocyte injection experiments suggested that muKSR plays a physiological role in the activation of the Ras signaling pathway in vertebrate cells. In order to investigate the mechanism of action of KSR in signal transduction, we examined the subcellular localization of KSR and the ability of KSR to interact with other proteins. To characterize KSR biochemically, we first generated a monoclonal antibody against an amino-terminal peptide of muKSR (Cys–Thr–Gln–Gln–Glu–Ile–Arg–Thr–Leu–Glu–Ala–Lys–Leu–Val–Lys). This antibody specifically recognized a band of approximately 105 kDa on western blots of murine fibroblast lysates and *Xenopus* oocyte lysates (Fig. 2a and data not shown), and this band was not visualized when antigenic peptide was added with the monoclonal antibody (data not shown). On some immunoblots, a doublet was noted at 105–108 kDa (data not shown). A commercial polyclonal anti-KSR antibody (Santa Cruz Biotech) raised against a different antigenic peptide also specifically recognized a band of 105 kDa (Fig. 2a). These results suggest that KSR migrates as a 105 kDa protein and that KSR is specifically recognized by these antibody preparations.

The intracellular localization of murine KSR was investigated using subcellular fractionation experiments. Murine fibroblasts were lysed in detergent-free buffer, and the lysates were separated by high-speed centrifugation into supernatant (S100) and pellet (P100) fractions and analyzed by immunoblotting using an anti-KSR polyclonal antibody: previous work had shown that cytoplasmic proteins are primarily found in the S100 fraction, whereas membrane-associated proteins are primarily localized in the P100 fraction [2,3]. Figure 2b demonstrates that although KSR was found primarily in the cytoplasmic (S100) fraction of serum-starved cells, it was found primarily in the membrane (P100) fraction of cells that had been stimulated with serum for 10 minutes. On some immunoblots, KSR in the membrane (P100) fraction displayed retarded mobility, consistent with a post-translational modification such as phosphorylation (data not shown). Using the same fractionation assay, previous work has demonstrated that the small GTP-binding protein rhoA translocates from the cytoplasm to the membrane at reproducible time points following serum-stimulation of cells [23]. Parallel experiments examining the subcellular localization of rhoA and KSR revealed that maximal levels

of both proteins were found in the membrane (P100) fraction after 10 minutes of serum stimulation (data not shown).

KSR binds to 14-3-3 in yeast and vertebrate cells

We next investigated the possibility that invertebrate and vertebrate forms of KSR bind to 14-3-3. The predicted *C. elegans* KSR protein (ceKSR) contains two amino-acid motifs related to the 14-3-3 binding motif Arg–Ser–X–Ser–X–Pro (Arg–Thr–Ser–Ser–Gly–Ser, amino acids 153–158, and Arg–Ser–Pro–Ser–Phe–Pro, amino acids 755–760) [17,18], and also contains a cysteine-rich CR-1 domain — a similar domain in Raf may be able to bind to 14-3-3 [6]. We examined the ability of ceKSR to interact with human 14-3-3 ζ using the yeast two-hybrid system. Full-length ceKSR interacted with 14-3-3 ζ but not with lamin, a generic control protein (Table 1). Like ceKSR, muKSR contains a CR-1 domain and two amino-acid motifs that are related to the 14-3-3 binding motif (Arg–Thr–Glu–Ser–Val–Pro, amino acids 389–394 and Arg–Arg–Leu–Ser–His–Pro, amino acids 825–840) [3], and two-hybrid analysis in yeast revealed that 14-3-3 ζ interacted with full-length muKSR but not with lamin (Table 1).

In order to determine whether KSR and 14-3-3 interact in vertebrate cells, co-immunoprecipitation experiments were performed using murine fibroblasts. Subconfluent NIH 3T3 cells were lysed, and muKSR immunoprecipitates were analyzed by immunoblotting using an anti-14-3-3 polyclonal antibody. This analysis revealed that 14-3-3 and muKSR formed a complex *in vivo* (Fig. 3a). To analyze the subcellular localization of the KSR/14-3-3 complex, we fractionated extracts from fibroblasts and separately analyzed the cytosolic (S100) and membrane (P100) fractions; anti-KSR immunoprecipitates of each fraction were analyzed by anti-14-3-3 immunoblotting, revealing that KSR and 14-3-3 formed a complex in both the cytoplasmic and membrane fractions of cultured fibroblasts (Fig. 3b).

Table 1

Detection of interactions between KSR and 14-3-3 proteins by two-hybrid screening.

	Transactivation domain fusion	
Binding domain fusion	14-3-3	ceKSR
14-3-3	+	+
muKSR	+	–
lamin	–	–

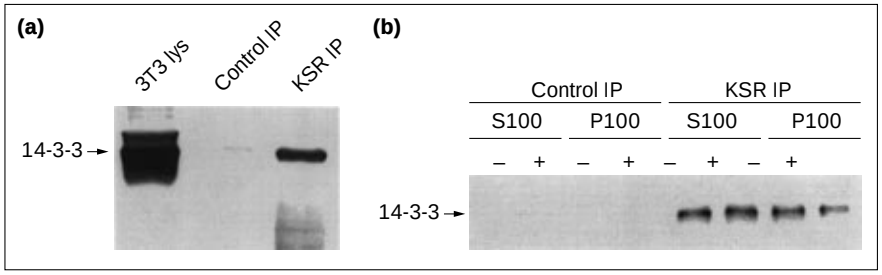
Yeast strain Y190 was co-transfected with plasmids encoding full-length muKSR or ceKSR and human 14-3-3 ζ (14-3-3), and grown in the absence of leucine and tryptophan. The efficacy of interactions was determined qualitatively by Xgal staining; lamin was used as a negative control.

KSR binds to Raf *in vitro* and *in vivo*

Many protein kinases are activated by homodimerization or heterodimerization: for example, the transforming growth factor-β (TGF-β) receptor family of serine/threonine kinases are activated by heterodimerization of Type I and Type II receptors [24]. Recent work suggests that Raf can also be activated by dimerization in cells [25,26]. Given that KSR is homologous to Raf, we investigated the possibility that KSR can bind to Raf.

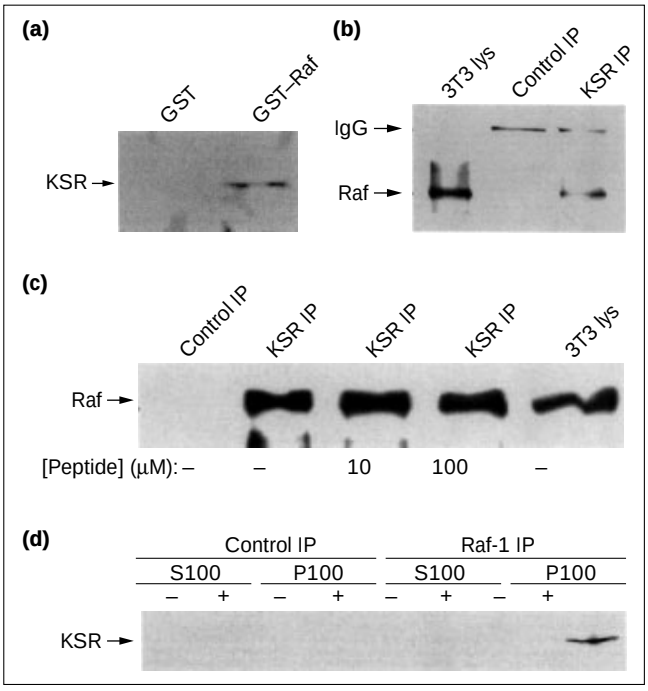
A fusion protein of glutathione S-transferase (GST) and Raf was produced in bacteria and used to determine if KSR interacts with Raf *in vitro*. KSR derived from NIH 3T3 cells bound to immobilized GST–Raf but not to GST protein alone (Fig. 4a). The ability of KSR to associate with Raf *in vivo* was tested by co-immunoprecipitation experiments; protein lysates derived from subconfluent NIH 3T3 cells were immunoprecipitated with anti-KSR. A western blot revealed that the immunoprecipitate contained Raf, suggesting that KSR and Raf form a complex *in vivo* (Fig. 4b). We recently demonstrated that serine-phosphorylated peptides based on sequences derived from Raf could disrupt

Figure 3



(a) Co-immunoprecipitation of muKSR and 14-3-3 from NIH 3T3 cells. Protein samples were analyzed by immunoblotting using an anti-pan-14-3-3 antibody. The samples were an extract of NIH 3T3 cells (3T3 lys), an immunoprecipitate obtained using a polyclonal anti-KSR antisera (KSR IP), and an immunoprecipitate obtained using a rabbit polyclonal anti-IgG antisera (control IP). The arrow indicates the band that corresponds to 14-3-3. (b) KSR/14-3-3 complex formation in membrane and cytosolic fractions. Quiescent (–) and serum-stimulated (+) NIH 3T3 cells were lysed in detergent-free buffer, and the extracts were separated by high-speed centrifugation to obtain cytosolic (S100) and membrane (P100) fractions. Immunoprecipitates obtained using a rabbit polyclonal anti-IgG antisera (control IP) and immunoprecipitates obtained using a polyclonal anti-KSR antibody (KSR IP) were analyzed using a polyclonal anti-14-3-3 antisera.

Figure 4



(a) Association of muKSR with Raf *in vitro*. NIH 3T3 cell lysates were added to immobilized GST–Raf fusion protein or GST alone and, after extensive washing, bound proteins were analyzed by immunoblotting using a polyclonal anti-KSR antibody. (b) Co-immunoprecipitation of KSR and Raf from NIH 3T3 cells. Protein samples (as described for Fig. 3a) were analyzed by immunoblotting using a polyclonal anti-Raf antibody. Arrows indicate the bands that correspond to Raf and to IgG added during immunoprecipitation. (c) Raf binding to KSR is not mediated by 14-3-3. Subconfluent NIH 3T3 cells were lysed and, in some instances, 10 μM or 100 μM pS-Raf-621 peptide (Leu–Pro–Lys–Ile–Asn–Arg–Ser–Ala–pSer–Glu–Pro–Ser–Leu–His–Arg, in which pSer is phosphoserine) was incubated with the lysate for 12 h at 4°C before immunoprecipitation. Protein samples (as described for Fig. 3a) were analyzed by immunoblotting using a polyclonal anti-Raf antibody. (d) Raf/KSR complex formation in serum-stimulated membrane fractions. Quiescent (–) and serum-stimulated (+) NIH 3T3 cells were lysed in detergent-free buffer, and the extracts were separated by high-speed centrifugation to obtain cytosolic (S100) and membrane (P100) fractions. Immunoprecipitates obtained using a rabbit polyclonal anti-IgG antisera (control IP) and immunoprecipitates obtained using a rabbit polyclonal anti-Raf antibody (Raf-1 IP) were analyzed using a monoclonal anti-KSR antisera.

complexes containing 14-3-3 [15]. In order to determine whether the interaction of Raf with KSR was mediated by 14-3-3, 100 μM pS-Raf-621 peptide [15] was added to fibroblast lysates, and anti-KSR immunoprecipitates were obtained. Addition of this peptide did not block the association of Raf with KSR (Fig. 4c); 14-3-3 binding to KSR was, however, inhibited. These results suggest that the association of Raf and KSR is not mediated by 14-3-3.

In order to analyze the subcellular localization of the Raf/KSR complex, we fractionated extracts from serum-starved and serum-stimulated fibroblasts and separately analyzed anti-Raf immunoprecipitates from the cytosolic (S100) and membrane (P100) fractions by anti-KSR immunoblotting. Raf and KSR only formed a complex in the membrane fraction of serum stimulated cells (Fig. 4d).

Discussion

Members of the recently discovered KSR family of proteins were shown to positively mediate Ras signaling in *C. elegans* and *Drosophila* [17–19], and homologous mammalian genes were identified on the basis of sequence similarity [19]; however, the physiological role of mammalian KSR in signal transduction is not well understood. Here, we have shown that muKSR has biological activity in *Xenopus* oocytes; overexpression of muKSR promoted GVBD, MAP kinase activation, and cdc2 kinase activation. A measurable response to KSR required co-expression of 14-3-3. The activity of KSR was blocked by co-expression of dominant-negative Raf, showing that the action of KSR requires functional Raf. Our results complement recent observations from Therrien *et al.* [27] who demonstrated that full-length KSR could stimulate *Xenopus* oocyte maturation in the presence of oncogenic Ras, but not alone. Taken together, these observations suggest that overexpression of KSR stimulates *Xenopus* oocyte maturation weakly, such that the effect of KSR can only be measured in oocytes that have been partially stimulated with a second molecule such as 14-3-3 or activated Ras. We conclude that KSR is likely to positively mediate Ras pathway signaling in vertebrate organisms.

Previous analyses of KSR have been largely genetic. Here, we have begun to investigate the biochemical characteristics of KSR; vertebrate KSR behaved as a 105 kDa protein that was largely localized to the cytoplasmic compartment of serum-starved cells and the membrane compartment of serum-stimulated cells. Invertebrate and vertebrate forms of KSR contain multiple potential 14-3-3 binding sites, and we found that invertebrate and vertebrate KSR can associate with 14-3-3 in yeast nuclei, and that vertebrate 14-3-3 associates with KSR in both the cytosolic and membrane fractions of cell lysates. The observation that both invertebrate and vertebrate KSR can associate with 14-3-3 protein suggests that this activity of KSR has been widely conserved and is therefore likely to be functionally significant. While our data do not conclusively establish the functional significance of the interaction between 14-3-3 and KSR, the oocyte experiments suggest that 14-3-3 may have a stimulatory effect on KSR activity. In addition, the ability of 14-3-3 protein to bind to both ceKSR and muKSR implies that this interaction is a conserved property of the entire family of KSR proteins.

Homodimerization was recently proposed as a potential mechanism for Raf kinase activation [25,26]; when Raf fusion proteins containing engineered dimerization domains are induced to dimerize, Raf kinase activity is

markedly stimulated. Several other serine/threonine kinases, including the TGF-β receptors, are activated by heterodimerization [24]. Here, we have shown that KSR could form a complex with Raf and that the Raf/KSR complex was present only in the membrane compartment of serum-stimulated cells. In contrast, the interaction between KSR and 14-3-3 occurred in both the cytoplasmic and membrane compartments of serum-starved and serum-stimulated cells. Previous work has similarly demonstrated that 14-3-3 is constitutively bound to Raf in both membrane and cytoplasmic compartments [10]. Our results complement findings of Therrien *et al.* [27] who demonstrated that KSR and Raf-1 formed a complex in *Drosophila* embryos and in co-transfected 293 cells.

Our results suggest that, in serum-starved cells, KSR is part of a complex that includes 14-3-3 and is primarily cytoplasmic; following serum stimulation, KSR translocates to the membrane and forms a complex with Raf. This raises a new and intriguing question: how is KSR translocated to the membrane? Although our data do not answer this question, it seems unlikely that Raf brings KSR to the membrane because Raf and KSR do not appear to associate in the cytoplasm. KSR, Raf and 14-3-3 all appear to positively regulate Ras signaling, and addition of KSR to the Ras/Raf/14-3-3 complex may have important functional consequences. One possibility is that the addition of KSR to the complex stabilizes the activated conformation of Raf. Alternatively, KSR may serve as a linker molecule that recruits substrates for Raf, rather like the Ste5 protein which tethers multiple MAP kinase cascade enzymes in budding yeast [28].

Materials and methods

Antibodies

Goat polyclonal anti-KSR antisera, rabbit anti-Raf antisera, and rabbit anti-ERK1 antisera were obtained from Santa Cruz Biotech. Agarose-conjugated $p13^{suc}$ was obtained from Upstate Biotechnology Inc. We generated a murine monoclonal anti-KSR antibody using a KLH-conjugated KSR peptide as an immunogen (Cys–Thr–Gln–Gln–Glu–Ile–Arg–Thr–Leu–Glu–Ala–Lys–Leu–Val–Lys). The KSR peptide and other peptides described in this work were synthesized using standard 9-Fluorenylmethyloxycarbonyl (FMOC) chemistry and purified by C_{18} reverse-phase high pressure liquid chromatography. Their identity was confirmed by amino-acid analysis as described previously [15]. Splenic lymphocytes from mice injected with the KSR peptide were fused to myeloma cells and fusion cell supernatants were tested by ELISA using the KSR peptide. The pS-Raf-621 peptide (amino-acid sequence Leu–Pro–Lys–Ile–Asn–Arg–Ser–Ala–pSer–Glu–Pro–Ser–Leu–His–Arg, in which pSer is phosphoserine) was generated and purified as described previously [15].

Yeast two-hybrid assays

The *C. elegans ksr-1* cDNA [17], predicted to encode amino acids 1–771, was inserted into the pGAD plasmid as an in-frame fusion with the transactivation domain of GAL4 (residues 768–881) [12]. The muKSR cDNA [19], predicted to encode amino acids 1–874, was inserted into the pAS1 plasmid as an in-frame fusion with the DNA-binding domain of GAL4 (residues 1–147) [12]. The human 14-3-3 ζ cDNA was inserted into both pGAD and pAS1 as described previously [12]. Yeast strain Y190 was co-transfected with pGAD-derived and pAS1-derived plasmids and yeast were plated on media lacking leucine and tryptophan. The β-galactosidase activity of co-transfected cells was assessed using Xgal as a substrate as described previously [12].

Protein analysis

NIH 3T3 cells and *Xenopus* oocytes were lysed using NP40 lysis buffer (0.5% NP40, 137 mM NaCl, 50 mM NaF, 5 mM EDTA, 10 mM Tris pH 7.5, 2 mM phenylmethylsulfonyl fluoride, 25 μM leupeptin, 0.2 U ml^{-1} aprotinin). Lysates were cleared by low-speed centrifugation and stored at –80°C [20]. For western blot experiments, proteins were separated by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and electrophoretically transferred to nitrocellulose filters. Filters were blocked with 5% nonfat dried milk and 1% bovine serum albumin in TBST (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 or horseradish-peroxidase-coupled secondary antibody and color-developing agents (Promega) or chemiluminescence-developing agents (ECL, Amersham).

For subcellular fractionation experiments, quiescent and serum-stimulated NIH 3T3 cells were lysed in detergent-free lysis buffer (137 mM NaCl, 50 mM NaF, 5 mM EDTA, 10 mM Tris pH 7.5, 2 mM phenylmethylsulfonyl fluoride, 25 μM leupeptin, 0.2 U ml^{-1} aprotinin), precleared by low-speed centrifugation (12 000 *g* for 5 min), and then separated by centrifugation at 100 000 *g* for 1 h. Supernatants (S100 fraction) were removed and pellets (P100 fraction) were resuspended in an equal volume of lysis buffer with 1% Triton X-100 [2,3].

For binding assays *in vitro*, cell lysates were added to immobilized recombinant GST fusion proteins. The entire coding region of the human Raf cDNA was subcloned into the bacterial expression vector pGEX-4T-3 (Pharmacia) and purified GST–Raf fusion protein was obtained using glutathione–agarose as described previously [15]. NIH 3T3 cell lysates were incubated with immobilized GST–Raf or GST protein at 4°C for 1 h. Immobilized proteins were washed extensively with lysis buffer containing added NaCl (final concentration 1 M). Gel sample buffer was added, and boiled samples were separated by SDS–PAGE, transferred to nitrocellulose membranes, and analyzed by immunoblotting.

For co-immunoprecipitation assays, protein-A-agarose or protein-G-agarose (Santa Cruz Biotech) was used to immobilize antibody-bound proteins. Immunoprecipitates were washed with lysis buffer containing added NaCl (final concentration 1 M), and analyzed by SDS–PAGE as above.

Xenopus *oocyte microinjection*

Purified plasmid DNA (pSP64T) containing inserts encoding muKSR, human14-3-3 ζ, human wild-type Raf, or human Lys375→Met mutated dominant-negative Raf (NAF) was linearized and used for transcription *in vitro* [20]. RNA transcribed *in vitro* (10–20 nl), at a concentration of 1 μg μl^{-1}, was injected into each oocyte. Some oocytes were stimulated with 8.25 μg ml^{-1} insulin.

Mature female frogs were used to obtain Stage VI fully grown immature oocytes as described previously [20]. Oocytes were maintained in 1× modified Barth's solution with HEPES, 1 mg ml^{-1} Ficoll 400, 1 mg ml^{-1} bovine serum albumin, and added antibiotics [20]. Injected oocytes were incubated at 19°C.

GVBD was assessed by looking for the presence of a broad white spot on the animal pole of the oocyte. Histone kinase assays were performed using $p13^{suc}$ beads to purify cdc2 from oocyte lysates [12]. After a 1 h incubation at 4°C, immobilized p13 was washed 3 times in NP40 lysis buffer containing added NaCl (final concentration 1 M). Kinase reactions were performed *in vitro* using kinase buffer (20 mM HEPES pH 7.5, 10 mM $MgCl_2$, 1 mM DTT, 100 μM ATP), and added Histone H1 and [^{32}P]ATP. Kinase reactions were terminated after

15 min by the addition of gel sample buffer. Reaction mixtures were analyzed by SDS–PAGE followed by autoradiography.

MAP kinase assays were performed using a polyclonal agarose-conjugated anti-MAP-kinase antisera (Santa Cruz Biotech) to purify MAP kinase from oocyte lysates. After a 1 h incubation at 4°C, anti-MAP-kinase immunoprecipitates were washed 3 times in NP40 lysis buffer containing added NaCl (final concentration 1 M). Kinase reactions were performed *in vitro* using kinase buffer (20 mM HEPES pH 7.5, 10 mM $MgCl_2$, 1 mM DTT, 100 μM ATP), and added myelin basic protein (Sigma) and [^{32}P]ATP [20]. Kinase reactions were terminated after 20 min by the addition of gel sample buffer. Reaction mixtures were analyzed by SDS–PAGE. Bands that corresponded to myelin basic protein were isolated and analyzed by scintigraphy.

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