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Kinase Suppressor of Ras Is Ceramide-Activated Protein Kinase

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Summary

A proline-directed serine/threonine ceramide-activated protein (CAP) kinase mediates transmembrane signaling through the sphingomyelin pathway. CAP kinase reportedly initiates proinflammatory TNFα action by phosphorylating and activating Raf-1. The present studies delineate kinase suppressor of Ras (KSR), identified genetically in Caenorhabditis elegans and Drosophila, as CAP kinase. Mouse KSR, like CAP kinase, renatures and autophosphorylates as a 100-kDa membrane-bound polypeptide. KSR overexpression constitutively activates Raf-1. TNFα or ceramide analogs markedly enhance KSR autophosphorylation and its ability to complex with, phosphorylate, and activate Raf-1. In vitro, low nanomolar concentrations of natural ceramide stimulate KSR to autophosphorylate, and transactivate Raf-1. Other lipid second messengers were ineffective. Moreover, Thr^{269}, the Raf-1 site phosphorylated by CAP kinase, is also recognized by KSR. Thus, by previously established criteria, KSR appears to be CAP kinase.

Introduction

The sphingomyelin pathway is an ubiquitous, evolutionarily conserved signaling system initiated by hydrolysis of the plasma membrane phospholipid sphingomyelin to generate ceramide (Spiegel at al., 1996). Sphingomyelin degradation is catalyzed by sphingomyelinase (SMase), a sphingomyelin-specific form of phospholipase C. Two forms of sphingomyelinase have been identified based on their pH optima. Acid SMase (pH optimum 4.5–5) resides in lysosomes (Kolesnick, 1991) and has also been identified in plasma membrane (Liu and Anderson, 1995). Neutral SMase (pH optimum 7.4) is either Mg^{2+}-dependent and membrane-bound or cytosolic and cation-independent (Spiegel et al., 1996). Both SMases hydrolyze the same phosphodiester bond to yield ceramide and phosphocholine. Once generated, ceramide acts as second messenger, mediating signaling for a variety of cellular stimuli.

Most, if not all, mammalian cells appear capable of signaling through the sphingomyelin pathway. Receptors as distinct as those for IL-1β, progesterone, γ-interferon, and tumor necrosis factor α (TNFα), as well as CD28, utilize the sphingomyelin pathway as a downstream effector system (for review, see Hannun, 1996; Jarvis et al., 1996; Spiegel et al., 1996). In this capacity, the sphingomyelin pathway appears to signal pleiotropic functions, inducing proliferation of fibroblasts, differentiation of promyelocytes, inhibition of the respiratory burst in human neutrophils, survival of T9 glioma cells, inhibition of insulin signaling through IRS-1, and apoptosis in numerous mammalian cell systems, to list a few. The most comprehensive studies on the involvement of the sphingomyelin pathway in signal transduction have been carried out for TNFα. Evidence has been provided that this pathway initiates both proinflammatory and apoptotic signaling for TNFα. Kronke and coworkers used mutants of the cytoplasmic region of the 55 kDa TNF receptor to demonstrate that specific receptor domains link to the different sphingomyelinases (Wiegmann et al., 1994). A membrane-proximal region linked the neutral SMase to the extracellular signal–regulated kinase (ERK) cascade and proinflammatory responses, while a carboxyl-terminal region containing the death domain connected to acid SMase.

A number of direct targets for the signaling action of ceramide have now been identified. These include a ceramide-activated protein kinase (CAP kinase) (Mathias et al., 1991; Joseph et al., 1993; Liu et al., 1994), a ceramide-activated protein phosphatase (Dobrowsky and Hannun, 1992; Hannun, 1996), and the protein kinase C isoform ζ (Lozano et al., 1994; Muller et al., 1995). The most well-defined target for ceramide action is CAP kinase. This enzyme is a 97 kDa Ser/Thr protein kinase that is exclusively membrane-associated. CAP kinase belongs to the family of proline-directed Ser/Thr protein kinases (Mathias et al., 1991; Liu et al., 1994), and its activity is enhanced by treatment of intact cells or isolated membranes with TNFα, IL-1β, ceramide analogs, and bacterial sphingomyelinases (Spiegel et al., 1996). CAP kinase is distinguished from other proline-directed protein kinases by its preference for X–Thr–Leu–Pro–X, a somewhat unusual variation of the minimal substrate motif recognized by this kinase family (Joseph et al., 1993).

Although the full range of the metabolic activities of CAP kinase is as yet unknown, recent studies have suggested that it signals the proinflammatory action of TNFα via activating Raf-1 (Yao et al., 1995). Raf-1 was first identified as the normal cellular counterpart of *v-raf*, the transforming gene of the murine sarcoma virus (Williams and Roberts, 1994). Two other related members of this family, *A-raf* and *B-raf*, were discovered subsequently (Storm et al., 1990; Stephens et al., 1992). Upon stimulation by growth factors, an N-terminal region of cytoplasmic Raf-1 binds to GTP-Ras, and Raf-1 is recruited to the plasma membrane, where it is activated by an unknown mechanism (Zhang et al., 1993; Leever et al., 1994). Active Raf-1 phosphorylates and stimulates a dual specificity kinase MEK1, which in turn phosphorylates and activates ERKs. Raf-1 mediates signal transduction induced by numerous growth factors (Rapp, 1991) and some cytokines including TNFα (Belka et al.,

1995). Recently, we reported that CAP kinase mediates TNF-induced Raf-1 activation at the plasma membrane of HL-60 cells. CAP kinase formed a complex with Raf-1, and phosphorylated Raf-1 in a ceramide- and TNF-dependent manner (Yao et al., 1995). CAP kinase phosphorylated Raf-1 on $Thr^{268,269}$, increasing Raf-1 activity toward MEK1, linking the TNF receptor to proinflammatory ERK targets such as phospholipase A_2 (Weigmann et al., 1994; Adam et al., 1996; Schatzman et al., unpublished data).

While activation of Raf-1 involves upstream binding to GTP-Ras, recent studies have identified the existence of a kinase suppressor of Ras (KSR) (Kornfeld et al., 1995; Sundaram and Han, 1995; Therrien et al., 1995). This putative protein kinase was isolated recently by selection and complementation of genetic mutations in Drosophila and Caenorhabditis elegans (Kornfeld et al., 1995; Sundaram and Han, 1995; Therrien et al., 1995). KSR appeared to function either upstream of Raf or in parallel with Raf in these systems (Downward, 1995). The predicted size of C. elegans and Drosophila KSR was about 90 kDa and 115 kDa, respectively, whereas the size of a murine homolog was about 100 kDa. A partial human cDNA has also been sequenced. The N-terminal regions of Drosophila and mammalian KSR contain four conserved domains, CA1–CA4. CA1 is a domain unique to KSR, CA2 is a putative Src homology 3 recognition site, CA3 is a cysteine-rich domain with similarity to the lipid-binding moiety of protein kinase C, and CA4 is a serine/threonine-rich domain that resembles the CR2 domain of Raf-1 (Therrien et al., 1995). In all species, the C-terminal region of KSR contains the 11 conserved kinase subdomains found in all known protein kinases. However, KSR lacks the signature sequences of any specific kinase group, although it is distantly related to the Raf family. KSR is, nonetheless, unlikely to be a Raf family member. The N-terminal Ras-binding domain (RBD), which is critical for Raf–Ras interaction, is absent from KSR. Furthermore, there was no interaction between Ras and KSR in the yeast two-hybrid system (Sundaram and Han, 1995; Therrien et al., 1995). In addition, kinase subdomain VIII, which is important for substrate recognition, is not conserved between KSR and Raf-1, suggesting that these kinases have different cellular targets. This was confirmed in the yeast two-hybrid system, which, as predicted, demonstrated strong interaction between Raf-1 and MEK, but not between KSR and MEK (Therrien et al., 1995). Whether KSR might be a tyrosine or serine/threonine kinase is also uncertain. The amino acid sequence YI(L)APE in subdomain VIII of KSR from all species resembles that of a Ser/Thr kinase rather than a tyrosine kinase, which usually contains the consensus sequence WXAPE. In contrast, both C. elegans and Drosophila KSR contain the HKDLR motif indicative of tyrosine kinases at subdomain VI, while both mammalian KSR possess the HKDLK motif typical of serine/threonine kinases. This implies that the mammalian KSR homologs might represent a distinct subgroup in a KSR superfamily. The mouse and partial human KSR display another interesting feature in kinase subdomain II, in which a conserved lysine residue involved in the phosphotransfer reaction in all mammalian kinases is substituted with arginine. This feature suggests that mammalian KSR might not even function as an active protein kinase. None of the isolated KSR cDNAs have yet been expressed and proven to be active protein kinases. In fact, KSR appeared to act in a kinase-independent manner when cooperating with oncogenic *ras* to promote Xenopus oocyte maturation and cellular transformation, since it associated with and activated Raf-1 in vivo, but did not phosphorylate Raf-1 in vitro (Therrien et al., 1996).

Despite the lack of biochemical information, the available genetic evidence strongly suggests a similarity between KSR and CAP kinase. KSR is either upstream or parallel to Raf-1 in genetic screens, is similar in size to CAP kinase, and contains a putative lipid-binding site. To evaluate potential similarities, we have compared KSR with CAP kinase biochemically. Here, we show that mouse KSR expressed in COS-7 cells, like CAP kinase, is an active protein kinase of 100 kDa that is exclusively membrane-bound and acts immediately upstream of Raf-1. Treatment of intact cells with TNFα or ceramide analogs markedly enhanced the ability of KSR to autophosphorylate, as well as to phosphorylate and activate Raf-1. Natural ceramide also stimulated these activities in vitro. Activation of KSR is specific for ceramide, as other lipid second messengers are ineffective. Moreover, the site on Raf-1, $Thr^{268,269}$, selectively phosphorylated by CAP kinase, is also recognized by KSR. These data strongly suggest that KSR is CAP kinase.

Results

Expression of KSR Leads to Constitutive Activation of Raf-1

Previous investigations (Mathias et al., 1991; Joseph et al., 1993; Liu et al., 1994; Yao et al., 1995) defined CAP kinase as a 97 kDa, membrane-associated, proline-directed, Ser/Thr protein kinase. CAP kinase activity is renaturable in an SDS gel and is enhanced by treatment of intact cells or isolated membranes with ceramide analogs. Recently, we showed that CAP kinase is upstream of Raf-1 and that its activity toward Raf-1 can be stimulated by ceramide and TNFα (Yao et al., 1995). Phosphorylation of Raf-1 by CAP kinase increases Raf-1 activity toward its physiological substrate MEK1. To determine if KSR might act similarly to CAP kinase, we cloned mouse KSR cDNA into a mammalian expression vector pcDNA 3 and tagged the cDNA at the N terminus with a Flag sequence. After transient expression of this construct in COS-7 cells, we probed both membrane and cytosolic fractions with anti-Flag antibody. As shown in Figure 1A, a single band of $\sim$100 kDa was detected in the membrane fraction from cells expressing the KSR construct but not the control vector. We next tested whether KSR had protein kinase activity. 5×10^6 COS-7 cells expressing Flag-tagged KSR were lysed with NP-40 buffer, and KSR was immunoprecipitated with anti-Flag antibody. Activity of KSR was assayed for autophosphorylation after renaturation in an SDS gel (Figure 1B). KSR was detected as a single autophosphorylating band of about 100 kDa in lysates from cells expressing KSR but not vector alone. These studies indicate that KSR is an active protein kinase.

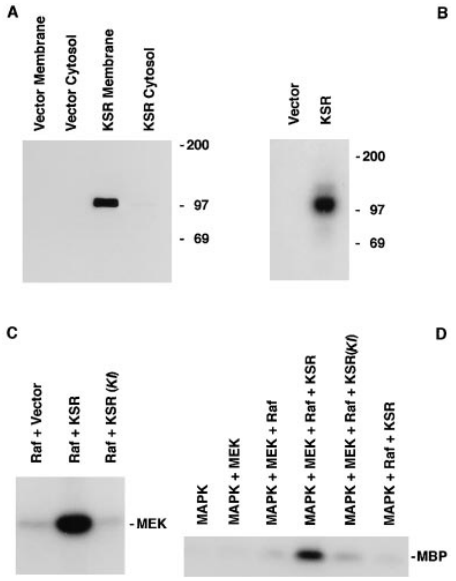

Figure 1. Expression of KSR, a 100 kDa, Membrane-Associated, Renaturable Protein Kinase, Leads to Activation of Raf-1

(A) COS-7 cells were transfected with Flag-tagged mouse KSR as described in Experimental Procedures and homogenized at 60 hr posttransfection. Equal amounts (30 μg) of microsomal membrane and cytosolic protein were resolved by SDS–PAGE, transferred onto a PVDF membrane, and probed with anti-Flag antibody.

(B) Flag-tagged KSR, immunoprecipitated from 2 mg of an NP-40 lysate of COS-7 cells with an agarose-conjugated anti-Flag antibody, was resolved by 7.5% SDS–PAGE, renatured, and autophosphorylated as described previously.

(C) COS-7 cells, cotransfected with Flag-tagged Raf-1 and pcDNA3 vector, or Flag-tagged Raf-1 and Flag-tagged KSR or kinase-inactive KSR, were lysed with RIPA buffer at 60 hr posttransfection. Flag-tagged Raf-1 was immunoprecipitated and incubated with 40 μl of reaction buffer A (40 mM Tris [pH 7.5], 10 mM $MgCl_2$, 30 mM NaCl) containing 0.6 μg of kinase-inactive MEK1 (K97M-MKK1), 50 μM ATP, and 30 μCi of [γ-^{32}P]ATP (3000 Ci/mmol) for 30 min at 22°C. The reaction was stopped with Laemmli buffer and phosphorylated MEK1 resolved with SDS–PAGE (7.5%) and autoradiography. Some studies used untagged KSR (n = 4) and yielded identical results. Expression levels of Raf-1 were similar in all samples as monitored by Western blot (not shown). These data represent one of three similar experiments.

(D) COS-7 cells were cotransfected and Flag-tagged Raf-1 was immunoprecipitated as in (C). Raf-1 activity was measured as in (C) using 1.25 μg of recombinant human MEK1 (Santa Cruz), 0.625 μg of recombinant human ERK1 (UBI), and MBP. After 30 min, phosphorylated MBP was resolved by 15% SDS–PAGE, prior to autoradiography. It should be noted that KSR/CAP kinase had almost no direct effect on MBP phosphorylation (not shown). These data represent one of three similar experiments.

To determine whether KSR might mimic CAP kinase in activating Raf-1 in vivo, Flag-tagged Raf-1 and KSR were coexpressed. Raf-1 was immunoprecipitated with anti-Flag antibody, and Raf-1 kinase activity was assayed using kinase-inactive MEK1 (K97M-MKK1) as substrate. As shown in Figure 1C, a marked increase in Raf-dependent MEK1 phosphorylation was detected in the sample cotransfected with Raf-1 and KSR, as compared to the sample from cells transfected with Raf-1 and control vector. It should be noted that KSR did not directly phosphorylate MEK1 (not shown). In contrast to wild-type KSR, kinase-inactive KSR(D683A/D700A) did not support Raf-1 activation. Similar results were obtained when Raf-1 activity was measured by reconstitution of the entire MAP kinase cascade (Figure 1D). Furthermore, dephosphorylation of immunoprecipitated Raf-1 using protein phosphatase 2A blocked Raf-1 signaling in either assay, and rephosphorylation with KSR/CAP kinase restored signaling (not shown). Thus, by three criteria—identical molecular weight, exclusive membrane-association, and renaturable protein kinase activity that activates Raf-1—KSR appeared highly similar to CAP kinase.

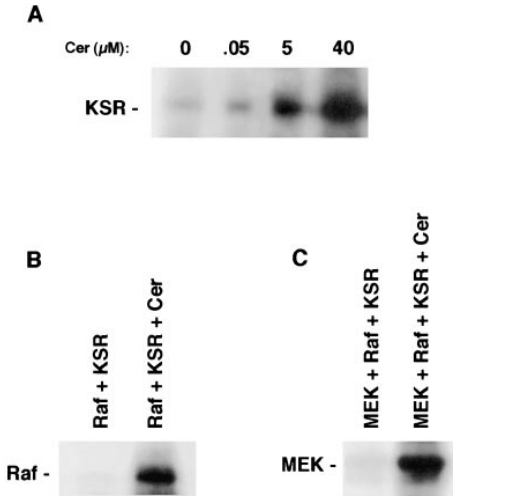

Figure 2. Ceramide Treatment of COS-7 Cells Increases the Ability of KSR to Autophosphorylate and to Phosphorylate and Activate Raf-1

(A) COS-7 cells, transfected as in Figure 1A, were incubated for 4 hr in serum-free medium and then treated for 10 min with C2 ceramide (Biomol). Cells were lysed with NP-40 lysis buffer, and Flag-tagged KSR immunoprecipitated and assayed for autophosphorylation in solution as described in Experimental Procedures. KSR is expressed at similar levels in each sample as monitored by Western blot (not shown). Data represent one of three similar experiments.

(B and C) COS-7 cells expressing Flag-tagged KSR were stimulated with 1 μM C2 ceramide for 10 min and lysed with NP-40 buffer as in (A). Flag-tagged KSR was immunoprecipitated and assayed for phosphorylation and activation of recombinant Raf-1 as described in Experimental Procedures. Data represent one of three experiments.

Activation of KSR by Ceramide and TNF In Vivo

To provide additional evidence that KSR might be CAP kinase, the effect of ceramide treatment on activation of KSR was tested. 2×10^6 COS-7 cells expressing KSR were treated with different doses of the ceramide analog, C2 ceramide, as indicated in Figure 2A. KSR was then immunoprecipitated with anti-Flag antibody and assayed for autophosphorylation in an immune complex kinase assay. Similar to what was observed in the in-gel assay shown in Figure 1B, KSR from resting cells manifested detectable basal activity. It should be noted that the exposure time for the autoradiograph in Figure 1B was 20 times longer than that in Figure 2A. Ceramide treatment induced dose-dependent enhancement of KSR-autophosphorylating activity. As little as

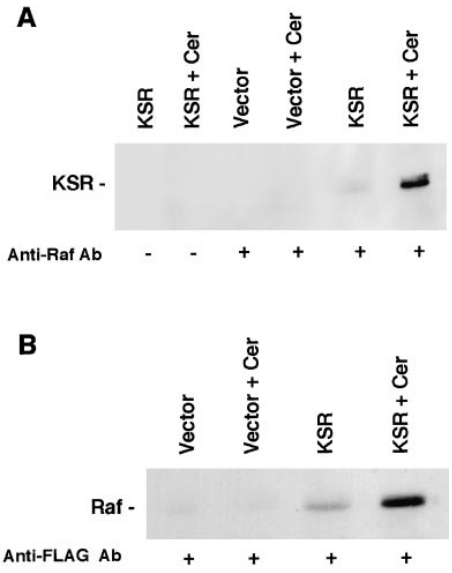

Figure 3. Ceramide Stimulates KSR to Complex with Raf-1.

(A) COS-7 cells, transfected with Flag-tagged KSR or pcDNA3 vector plasmid, were treated with 50 μM C2 ceramide for 5 min and lysed with NP-40 buffer. Lysates were subjected to 4 hr of immunoprecipitation with anti-Raf-1 antibody. Two controls, KSR and KSR with ceramide treatment, received protein A–conjugated Sepharose beads without anti-Raf-1 antibody. KSR complexed to Raf-1 was resolved by SDS–PAGE and detected by Western blot using anti-Flag antibody as in Figure 1A. Data represent one of four similar experiments.

(B) COS-7 cells, cotransfected with FLAG-tagged KSR and Raf-1 or vector and Raf-1, were handled as in (A), except immunoprecipitation was performed with anti-Flag antibody and Western blot with anti-Raf-1 antibody. Data represent one of three similar experiments.

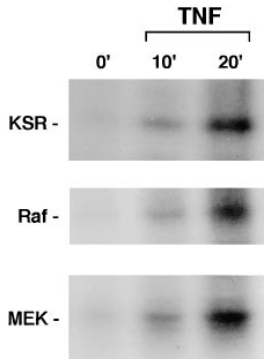

Figure 4. TNF Treatment of COS-7 Cells Activates KSR

Flag-tagged KSR, immunoprecipitated from COS-7 cells treated with 10 nM TNFα, was assayed for autophosphorylation and the ability to phosphorylate and activate recombinant Raf-1, as described in Experimental Procedures. Data represent one of three similar experiments.

50 nM C2 ceramide induces an increase in KSR activity, and a maximal 5- to 10-fold effect was achieved with 40 μM C2 ceramide. This is the same range of ceramide concentrations previously shown to increase CAP kinase activity in HL-60 cells (Liu et al., 1994).

We next examined the effect of ceramide on KSR activity toward recombinant Raf-1 in an immune complex kinase assay. For these studies, KSR, immunoprecipitated from control or ceramide-treated cells, was incubated with a recombinant Raf-1 substrate that had been coexpressed with Ras and a protein tyrosine kinase Lck in Sf9 insect cells, as previously described (Yao et al., 1995). We titrated the amount of the Raf-1 used in the assay so that in the absence of KSR, Raf-1 autophosphorylation and Raf-1-dependent MEK1 (K97M-MKK1) phosphorylation were not detectable (not shown). Under these conditions of limiting substrate, minimal phosphorylation of both Raf-1 and MEK1 were visualized after a 30 min incubation with KSR immunoprecipitated from untreated cells (Figures 2B and 2C). Treatment of COS-7 cells with C2 ceramide (1 μM) markedly enhanced the activity of immunoprecipitated KSR to phosphorylate (Figure 2B) and activate Raf-1 (Figure 2C). Similar results were obtained using recombinant human Raf-1 singly expressed in and immunoprecipitated from COS-7 cells (not shown).

To understand the molecular mechanism of ceramide-dependent activation of Raf-1 by KSR, we tested whether Raf-1 and KSR form a protein complex. For these studies, lysates from cells expressing Flag-tagged KSR or control vector were immunoprecipitated with anti-Raf antibody or control beads (protein A–conjugated Sepharose beads) and then probed with anti-Flag antibody. KSR is readily detected in the sample expressing KSR and is immunoprecipitated with anti-Raf-1 antibody, but not with control beads (Figure 3A). Coimmunoprecipitation of KSR with Raf-1 was significantly enhanced when Raf-1 was immunoprecipitated from cells treated with 50 μM C2 ceramide. Similarly, when KSR was immunoprecipitated with anti-Flag antibody from a sample coexpressing Raf-1 and Flag-tagged KSR, Raf-1 was readily detected in the immunoprecipitated sample (Figure 3B). Again, coimmunoprecipitation of Raf-1 with KSR was dramatically enhanced by C2 ceramide treatment. Immunoprecipitated KSR also bound recombinant Raf-1 in vitro (not shown). A similar ceramide-dependent association of Raf-1 with CAP kinase was previously demonstrated (Yao et al., 1995), further strengthening the notion that KSR is CAP kinase.

Previous results indicated that CAP kinase is involved in TNFα-mediated signal transduction and its activity enhanced by TNF treatment (Liu et al., 1994; Yao et al., 1995). We therefore stimulated 2×10^6 COS-7 cells expressing KSR with TNFα (10 nM) and then assayed the autophosphorylating activity of KSR, as well as its activity to phosphorylate and activate recombinant Raf-1, as described in Figure 2. Figure 4 shows that TNF induced time-dependent enhancement of the autophosphorylating activity of KSR. Phosphorylation of recombinant Raf-1, as well as Raf-1 activity toward kinase-inactive MEK-1 (K97M-MKK1), were also stimulated dramatically by TNFα treatment. A maximal effect was achieved by 20 min of TNFα treatment, which correlates closely with the time course of the TNF-induced activation of CAP kinase previously shown in HL-60 cells (Liu et al., 1994; Yao et al., 1995). Kinase-inactive KSR from TNF-treated cells, however, did not display autophosphorylating or transactivating properties (not shown). In separate studies, TNFα, like ceramide (see Figure 3), induced KSR to complex with Raf-1 (not shown).

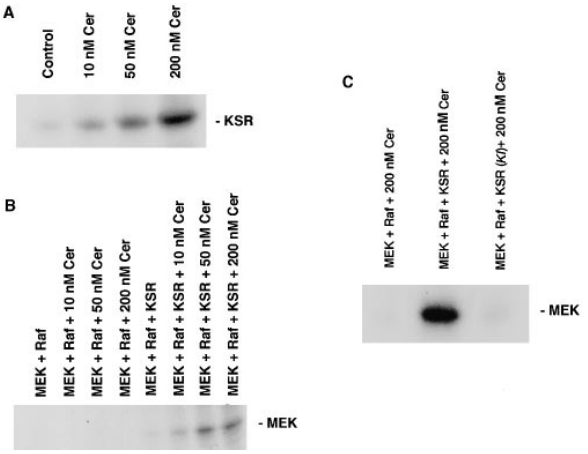

Figure 5. Ceramide Stimulates KSR Autophosphorylation and KSR-Dependent Raf-1 Activation In Vitro

(A) Flag-tagged KSR was immunoprecipitated as described in Experimental Procedures and assayed for autophosphorylation in 40 μl of reaction buffer B containing 10 mM $MnCl_2$, 10 μM ATP, and 30 μCi of [γ-^{32}P]ATP (3000 Ci/mmol) for 30 min at 22°C in the presence of natural ceramide from bovine brain (Avanti) or diluent. Phosphorylated KSR was resolved and detected as in Figure 2A. These data represent one of three similar experiments.

(B) Immunoprecipitated Flag-tagged KSR was assayed for activation of recombinant Raf-1 in vitro using kinase-inactive MEK1 (K97M-MKK1) as in Experimental Procedures in the presence or absence of bovine brain ceramide (Avanti). These data represent one of three similar experiments.

(C) The capacity of wild-type or kinase-inactive KSR to activate recombinant Raf-1 in vitro was determined as in (B). These data represent one of two similar experiments.

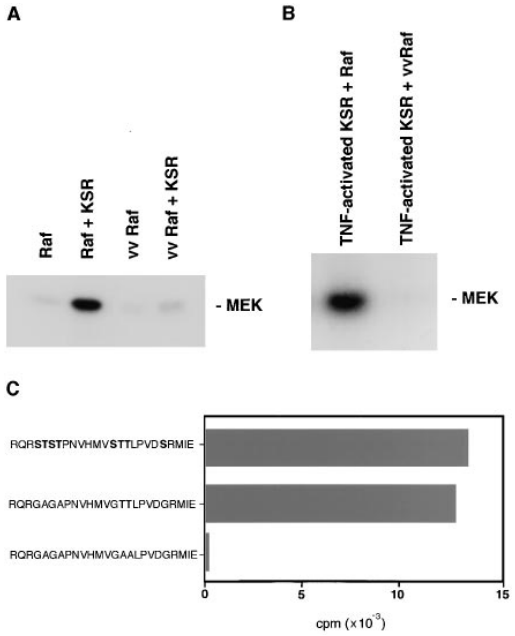

Figure 6. Raf-1 Activation by KSR Requires Thr^{268,269}

(A) KSR was coexpressed with Flag-tagged Raf-1 or vvRaf-1, and the activity of immunoprecipitated Raf-1 was measured using K97M-MKK1 as in Figure 1C. The expression levels of Raf-1 and vvRaf-1 were similar as monitored by Western blot (not shown). These data represent one of two similar experiments.

(B) Flag-tagged KSR was immunoprecipitated from a lysate of COS-7 cells treated for 20 min with 10 nM TNFα. KSR activity toward recombinant human Raf-1 or vvRaf-1 (a gift from Debbie Morrison) was measured using kinase-inactive MEK1 as described in Experimental Procedures. These data represent one of three similar experiments.

(C) Flag-tagged KSR was assayed with synthetic Raf-1 peptides as described in Experimental Procedures. The wild-type peptide contains eight potential phosphorylation sites (shown in bold). Substitution of alanines and glycines for serines and threonines generated one peptide (TTLP peptide) containing only two potential phosphorylation sites (Thr^{268,269}) and another peptide (AALP peptide) with no potential phosphorylation sites. These data represent one of three similar experiments.

Activation of KSR by Ceramide In Vitro

Subsequent studies evaluated the direct effect of ceramide on KSR activity in vitro. For these studies, KSR was immunoprecipitated from COS-7 cells and assayed for autophosphorylation and activation of Raf-1 in the presence or absence of natural ceramide. As shown in Figure 5A, autophosphorylation of KSR was enhanced in a dose-dependent manner by natural ceramide. As little as 10 nM natural ceramide was effective, and a maximal 10-fold effect was achieved with 200 nM. It should be noted that the effective concentration range for natural ceramide is about 100 times lower than the range for the synthetic C2 ceramide analog used in vivo. Other lipids including arachidonic acid, lyso-phosphatidic acid, phorbol 12-myristate 13-acetate, 1,2-diacylglycerol, phosphatidylserine, and phosphatidylcholine had no effect on activation of KSR (not shown).

Similarly, natural ceramide enhanced KSR activity toward Raf-1 in vitro. As shown in Figure 5B, in the absence of KSR, Raf-1 activity toward its physiological substrate MEK1 was barely detectable under the conditions of our assay. Addition of KSR induced phosphorylation of Raf-1 (not shown) and stimulated Raf-1 to phosphorylate MEK1 (Figure 5B). This activity was markedly enhanced by ceramide. The effect of ceramide was not due to the direct activation of Raf-1, since in the absence of KSR, the activity of Raf-1 was not stimulated by ceramide. Furthermore, dephosphorylation of KSR-activated Raf-1 using PP2A blocked Raf-1 signaling (not shown). In contrast to wild-type KSR, kinase-inactive KSR did not support ceramide-stimulated signaling (Figure 5C). Similar to CAP kinase (Yao et al., 1995), KSR had no direct effect on MEK or ERK (not shown). Nevertheless, in separate studies (n = 5), ceramide-activated KSR significantly increased phosphorylation and activation of ERK1 when added with recombinant Raf-1 and MEK1 (not shown). Thus, KSR signaling reconstitutes the entire ERK cascade in vitro. Identical results were obtained using recombinant human Raf-1 immunoprecipitated from COS-7 cells (not shown).

Substitution of Thr^{268,269} of Raf-1 Blocks Activation by KSR

Previous investigations showed that CAP kinase prefers the unusual motif –TLP– (Joseph et al., 1993). We mapped the CAP kinase phosphorylation sites on Raf-1 to a threonine doublet at Thr^{268} and Thr^{269} (Yao et al., 1995). Use of synthetic peptides derived from the primary Raf-1 sequence surrounding Thr^{268,269} with single or

double amino acid substitutions of alanine for threonine revealed that Thr[269] is the primary target for CAP kinase. Once Thr[269] is phosphorylated, CAP kinase then phosphorylates Thr[268] (Yao et al., 1995).

To define the relevance of Thr[268] and Thr[269] to activation of Raf-1 by KSR/CAP kinase, we used Raf-1 substituted with valine residues at these positions. In contrast to wild-type Raf-1, vvRaf-1 is not activated when coexpressed with KSR in COS-7 cells (Figure 6A). Furthermore, neither TNF nor ceramide treatment activates vvRaf-1 in COS-7 cells (not shown). However, vvRaf-1, like wild-type Raf-1, is activated 5–10-fold by EGF and the phorbol ester 12-*O*-tetradecanoylphorbol 13-acetate (TPA) (not shown). Similarly, recombinant vvRaf-1 from Sf9 cells is neither phosphorylated (not shown) nor activated in vitro by KSR/CAP kinase (Figure 6B). Identical results were obtained using wild-type Raf-1 and vvRaf-1 singly expressed in and immunoprecipitated from COS-7 cells (not shown). Additional studies used Raf-1 preparations with substitutions of valine residues for either Thr[268] or Thr[269]. When coexpressed, KSR/CAP kinase activated Raf-1(T268V) as effectively as wild-type Raf-1, whereas Raf-1(T269V) was not activated (not shown). Thus, Thr[269], but not the putative autophosphorylation site Thr[268] (Morrison et al., 1993), appears necessary for Raf-1 activation by KSR/CAP kinase.

To further evaluate KSR signaling, we assayed phosphorylation of a set of peptides derived from the primary amino acid sequence of Raf-1. As shown in Figure 6C, the wild-type Raf peptide, which contains eight potential phosphorylation sites, was phosphorylated to the same extent as the mutated Raf peptide containing only Thr[268] and Thr[269] (termed TTLP peptide). Hence, these other potential phosphorylation sites are not recognized by KSR. Furthermore, substitution of a glycine in the TTLP peptide in the position corresponding to proline 271 of Raf-1 reduced phosphorylation by 75%. As expected, the control peptide in which all serine and threonine residues were substituted with alanine and glycine residues (termed AALP peptide) showed negligible ^{32}P labeling. These studies indicate that KSR, like CAP kinase, recognizes Thr[268]/Thr[269]. It should be noted that identical results were obtained when phosphorylated peptide was resolved by a phosphocellulose paper assay or by reverse-phase high performance liquid chromatography.

All subsequent studies used the TTLP variant of Raf-1 peptide. To further evaluate the effect of ceramide on KSR activity, KSR was incubated with 30 nM natural ceramide in the presence of the TTLP peptide. Figure 7A shows that KSR activity toward this Raf-1 peptide was enhanced by ceramide in a time-dependent manner. The increase in activity appeared biphasic; a maximal 8-fold increase ($p < 0.05$ versus control) was detected after 5 min. Ceramide activation of KSR toward Raf peptide was also dose dependent at 45 min (Figure 7B). The maximal effect was achieved with 30 nM natural ceramide, which correlates closely to the effective concentrations that activated KSR toward Raf-1 in vitro, as shown in Figure 5B.

The effect of ceramide on the activation of KSR toward Raf peptide was specific. All other lipid second messengers tested at an equimolar concentration (30 nM) (including arachidonic acid [AA], lyso-phosphatidic acid [LPA], phorbol 12-myristate 13-acetate [PMA], and 1,2-diacylglycerol [DAG]) had no effect on activation of KSR in vitro (Figure 7C). Furthermore, recognition of the Raf peptide substrate by KSR was also specific. Figure 7D shows that KSR, like CAP kinase (Joseph et al., 1993), displayed minimal activity toward substrates recognized by other Ser/Thr kinases, including cAMP-dependent protein kinase A (PKA), S6 kinase, casein kinase II (CK II), calcium/calmodulin–dependent protein kinase II (CaM kinase II), and protein kinase C (PKC).

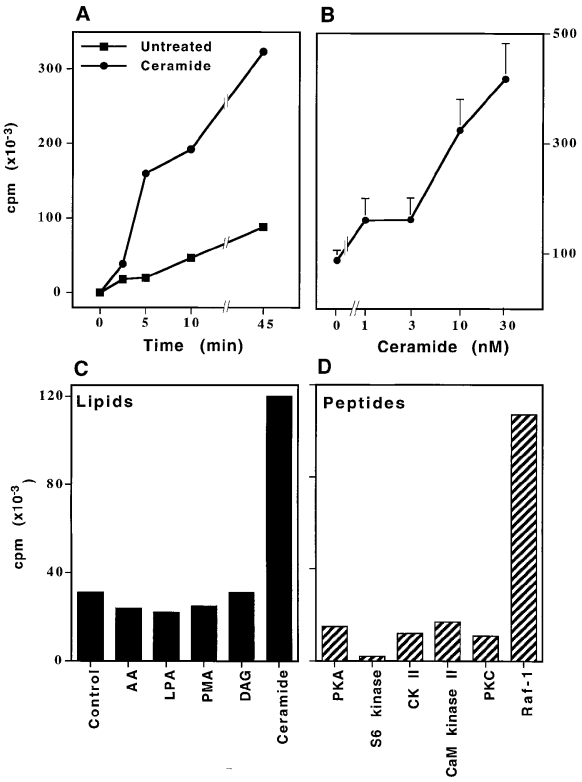

Figure 7. Ceramide Stimulates KSR Activity toward Raf-1 Peptide In Vitro

(A) Immunoprecipitated Flag-tagged KSR was assayed with TTLP or AALP peptides in the presence of 30 nM ceramide (Avanti) or diluent (DMSO). Background counts per minute (cpm), derived from samples incubated with the AALP peptide, which contains no phosphorylation sites, represented less than 25% of the untreated total and were subtracted from all points. These data represent one of three similar experiments.

(B) Immunoprecipitated Flag-tagged KSR was assayed with TTLP or AALP peptides in the presence of ceramide or diluent for 45 min as described in (A). These data represent one of four similar experiments.

(C) Immunoprecipitated Flag-tagged KSR was assayed for 20 min as in (A) in the presence of 30 nM lipid second messengers: (AA), arachidonic acid; (LPA), lyso phosphatidic acid; (PMA), phorbol 12-myristate 13-acetate; DAG: 1,2-diacylglycerol. These data represent one of three similar experiments.

(D) Immunoprecipitated Flag-tagged KSR was assayed for 5 min in the presence of 30 nM ceramide as in (A) using 0.3 mM Raf-1 peptide (TTLP peptide) or an equivalent amount of substrate peptides specific for other Ser/Thr protein kinases including cAMP-dependent protein kinase (PKA), S6 kinase, casein kinase II (CK II), calcium/calmodulin–dependent protein kinase (CaM kinase II), and protein kinase C (PKC). These data represent one of two similar experiments.

Discussion

The present studies demonstrate that recombinant KSR displays all of the previously recognized properties of CAP kinase. The molecular size of KSR is virtually identical to CAP kinase, and KSR renatures in an SDS gel. Furthermore, KSR, like CAP kinase, appears almost exclusively membrane associated. This subcellular localization is consistent with activation by ceramide, as ceramide is quite hydrophobic and, once generated, likely to remain within membranes. In fact, KSR immunoprecipitated from resting cells demonstrates modest basal activity, which is acutely enhanced by ceramide stimulation, indicative of signaling function. In addition to activation in vivo by treatment of cells with ceramide analogs, TNFα also stimulated KSR to autophosphorylate and transactivate Raf-1. Like CAP kinase, it appears that the mechanism by which KSR induces Raf-1 activation involves the formation of a protein complex and Raf-1 phosphorylation by KSR. Although interaction of KSR/CAP kinase and Raf-1 may be direct, involvement of an intermediary protein remains possible. In vitro, ceramide also induces KSR to autophosphorylate and to bind and activate Raf-1. Furthermore, CAP kinase and KSR recognize the same unusual proline-directed site, allowing for the development of a simple peptide assay for measuring activity. The fact that other lipid second messengers, including the structural homolog dihydroceramide and the backbone lipid sphingosine (not shown), fail to activate KSR points to a high degree of specificity for the structure of ceramide in the activation process. In sum, by every criterion we have previously established, KSR appears to be CAP kinase.

The designation of KSR as CAP kinase allows for more precise molecular ordering of transmembrane signaling events coupling the 55 kDa TNF receptor to proinflammatory signaling via the MAP kinase cascade. The currently available data suggest that an 11 amino acid domain (aa 309–319) of the cytoplasmic portion of the TNF receptor serves as a recognition site for an adaptor protein termed factor associated with neutral sphingomyelinase activation (FAN) (Adam-Klages et al., 1996). FAN-binding to the receptor specifically integrates signaling through to neutral sphingomyelinase in the plasma membrane. Ceramide is thus generated and stimulates KSR/CAP kinase to complex with and phosphorylate Raf-1, initiating signaling down the ERK cascade. One result of signaling via this mechanism is the activation by ERK of cPLA2 and the release of arachidonic acid (Weigmann et al., 1994).

The involvement of KSR/CAP kinase in signaling of other TNF-stimulated responses, such as proliferation, differentiation, NFκB activation, and apoptosis, has not been evaluated. Perhaps CAP kinase utilizes different targets to effect these distinct cellular responses. Alternatively, CAP kinase may represent a family of protein kinases, and each member may display a different substrate specificity. In this regard, many mammalian cells show, in addition to the 100 kDa form, membrane-associated, renaturable kinase activities of 90 kDa and 110 kDa that are stimulated by ceramide and TNFα in vivo (R. K., unpublished data). Whether these three protein kinases are alternatively spliced or posttranslationally modified products of a single gene or of multiple genes will require additional investigation. Consistent with this paradigm, three N-terminal splice variants of human KSR were found (Therrien et al., 1995). The availability of reagents to study the roles of KSR/CAP kinase in signal transduction should permit evaluation of these and other questions in the near future.

A unique feature of murine and human KSR is the substitution of an arginine for the conserved lysine in kinase subdomain II. This residue has been shown to be involved in the phosphotransfer reaction in numerous protein kinases and, in fact, substitution of this lysine with arginine results in abolition of the catalytic activity of several protein kinases (Hanks et al., 1988). The present investigations provide evidence that the lysine in this position is not mandatory. Structural elements of KSR/CAP kinase provide additional clues to its mechanism of activation. There is no signal sequence in KSR/CAP kinase that might permit membrane association. However, hydropathy plots define the carboxyl terminus as a highly hydrophobic region that might serve to interact with the membrane. Ceramide, once generated, may bind to the region CA3, which is homologous to the lipid-binding site of protein kinase C. Consistent with this proposal, ceramide has been shown to directly activate the ζ isoform of protein kinase C (Lozano et al., 1994; Muller et al., 1995). Preliminary studies suggest that autophosphorylated KSR/CAP kinase is the active form toward exogenous substrate (Yao et al., 1995). It is plausible that once ceramide induces KSR/CAP kinase autophosphorylation, KSR/CAP kinase becomes capable of phosphorylating Raf-1. Ceramide may also play a direct role in Raf-1 activation since Raf-1, which contains a putative lipid-binding site in region CR1, has recently been shown to be a ceramide-binding protein (Huwiler et al., 1996). In this capacity, ceramide binding to Raf-1 might function in Raf-1 translocation to the membrane compartment.

The proposed sequence of events suggests an alternative model for Raf-1 activation. The well-established paradigm for Raf-1 activation through tyrosine kinase receptors, involving adaptor proteins that contain Src homology domains and the activation of Ras, does not appear to play a role in TNF-induced Raf-1 activation. In this regard, the 55 kDa TNF receptor is not a tyrosine kinase and, hence, would not be expected to link to the set of adaptor proteins promoting Ras activation. Furthermore, Thr^{269} comprises a unique recognition site not previously shown to be involved in Raf-1 activation by tyrosine kinases. Nevertheless, Ras may play a role in TNF-induced Raf-1 activation. Preliminary studies show that N17Ras blocked KSR/CAP kinase–induced Raf-1 activation in COS-7 cells and that electroporation of anti-ras antibody Y13-259 abolished TNF-induced ERK1 activation in HL-60 cells. This information is consistent with the genetic data placing KSR downstream of Ras. Since KSR/CAP kinase does not bind Ras in the yeast two-hybrid assay (Sundaram and Han, 1995; Therrien et al., 1995), it is likely that KSR/CAP kinase affects Ras action indirectly through its role in modification of Raf-1 function. Whether another mechanism exists by which tyrosine kinase receptors can also utilize CAP kinase for signaling through Raf-1 is presently unknown. However,

preliminary data show that EGF does not activate KSR/CAP kinase in COS-7 cells and that maximal concentrations of EGF and TNF stimulate more than additive Raf-1 activation. Furthermore, EGF and TPA activate valine-substituted Raf-1 as effectively as wild-type Raf-1. These data suggest that TNF and EGF activate Raf-1 by different mechanisms. It should be noted that prior studies showed that dominant-negative Ras N17 had no effect on TNF-induced, stress-activated protein kinase (SAPK)/c-Jun kinase (JNK) activation in PC12 and COS-7 cells (Minden et al., 1994; Coso et al., 1995). Thus, the upstream elements linking TNF to the ERK and SAPK/JNK cascades are likely to be distinct.

The present studies define KSR as CAP kinase. This designation will likely impact three distinct areas of cell biologic research. The demonstration of alternative signaling mechanisms for activation of Raf-1 should provide a basis for new investigations into its role in various cellular responses, including inflammation, proliferation, and the regulation of anti-apoptosis. Furthermore, the availability of CAP kinase as a reagent should facilitate studies of mechanisms by which ceramide activates cellular targets to initiate transmembrane signaling. Lastly, the recognition that KSR/CAP kinase is integral to inflammatory signaling through the TNF receptor provides a foundation for studies into its role in the pathogenesis of TNF-mediated disease. It may also provide a biochemical target for pharmacologic manipulation of TNF action in vivo, with potential for clinical application.

Experimental Procedures

Cell Culture

COS-7 cells were grown in DMEM medium containing 10% fetal bovine serum (GIBCO BRL) at 37°C in a 5% CO_2 atmosphere. Cell number and viability were assessed by trypan blue exclusion analysis.

Construction of Mammalian Expression Vectors of Raf-1 and KSR

Construction of Flag–Raf

An 8 aa Flag epitope tag was introduced at the N terminus of human Raf-1 in pBluescript (ATCC, Rockville, MD) by the polymerase chain reaction (PCR) using CGG GGT ACC GCC ACC ATG GAC TAC AAG GAC GAC GAT GAC AAG GAG CAC ATA CAG GGA GCT TGG AAG as the 5′ primer and an internal Raf-1 sequence as the 3′ primer (GAA GGC AAG CTT CAG GAA C). The 470 bp PCR fragment was then exchanged with the native N-terminal Raf-1 sequence by digestion at the flanking KpnI/HindIII sites. The 1.9 kb complete Flag–Raf coding sequence was subcloned from pBluescript into pcDNA3 (Invitrogen) using the flanking KpnI/XbaI sites.

Raf-1 mutants, substituted with valine residues at positions 268, 269, or both, were generated in Flag–Raf by overlap extension PCR (Ho et al., 1989) using three mutagenic forward primers GTC CAC ATG GTC AGC GTC ACG CTA CCG GTG GAC AGC AGG ATG, CAC ATG GTC AGC ACC GTG CTA CCG GTG GAC AGC AGG ATG, and GTC CAC ATG GTC AGC GTC GTG CTA CCG GTG GAC AGC AGG ATG, respectively, and the upstream and downstream reverse primers CAG CAG TTT GGC TAT CAG C and CCC CAT GAA AAG CAG AAT G, respectively. All mutant PCR fragments were subcloned into Flag–Raf via internal BsgI and Eco47III restriction sites.

Construction of Flag–KSR

An oligonucleotide containing the Flag sequence, AAG CTT CCA GCA GCC ATG GAC TAC AAG GAC GAC GAT GAC AAA GCG AAT TCC, was first cloned into pcDNA3 through HindIII and EcoRI sites to generate pFlag–cDNA3. A KSR-containing EcoRI fragment was excised from pMA57 (a kind gift from Dr. Gerald Rubin) and cloned into pFlag–cDNA3, generating an in-frame fusion of Flag to the N terminus of the mouse KSR gene.

Substitutions of alanine residues for two conserved aspartates, D683 and D700, putatively involved in phosphotransfer, were introduced by two separate sets of overlap extension PCR (Ho et al., 1989). For initial introduction of the D683A mutation, the mutagenic forward primer AAA GCG CTC AAG TCC AAG AAT GTC TTC TAT G and reverse primer CTT GGA CTT GAG CGC TTT GTG CAC GAT GCC TTT TG were used to generate two PCR fragments from Flag–KSR with upstream primer CTC CCA TGG ACA TGC TTT CCT CGC and downstream primer CCA GAT CAA GGC CTC AGC AGG CTG. The two PCR fragments were then used as templates to produce a contiguous fragment using the same flanking primers. This fragment was subsequently used as the template for introducing the D700A mutation using the mutagenic forward primer ATC ACA GCC TTC GGG CTG TTT GGG ATC and reverse primer CAG CCC GAA GGC TGT GAT GAC CAC TTT GCC in two sequential PCR reactions as above. The final D683A /D700A double mutation PCR fragment was subcloned into Flag–KSR via internal BamHI and SunI restriction sites.

Expression of KSR and Raf-1 in COS-7 Cells

COS-7 cells were transfected using LipofectAMINE (GIBCO BRL) according to the manufacturer's instructions. At 60 hr posttransfection, cells were harvested in NP-40 lysis buffer (25 mM Tris [pH 7.5], 137 mM NaCl, 10% glycerol, 1% NP-40, 2 mM EDTA, 1 mM PMSF, 10 μg/ml leupeptin/soybean trypsin inhibitor, 5 mM $NaVO_4$). The homogenate was centrifuged at 6000 × g for 5 min, the supernatant collected, and protein content measured using BCA Reagent A (Pierce). Protein expression was monitored by Western blot as described (Yao et al., 1995).

Immunoprecipitation of KSR and Raf-1

Flag-tagged proteins were quantitatively immunoprecipitated from the COS-7 lysate using agarose-conjugated anti-Flag antibody (Scientific Imaging Systems) at 4°C for 4 hr or overnight. The beads were washed three times with NP-40 lysis buffer and one time with reaction buffer B (30 mM HEPES [pH 7.4], 5 mM $MgCl_2$, 1 mM DTT) before measuring kinase activity.

KSR Autophosphorylation

KSR was immunoprecipitated, washed as above, and incubated with 40 μl of reaction buffer B containing 10 mM $MnCl_2$, 10 μM ATP, and 30 μCi of [γ-^{32}P]ATP (3000 Ci/mmol) for 30 min at 22°C. The reaction was stopped by addition of Laemmli buffer. Samples were resolved by 7.5% SDS–PAGE and autoradiographed.

For some studies, KSR was assayed for in-gel autokinase activity after denaturation and renaturation as described previously (Liu et al., 1994).

Determination of Raf-1 Activity

Raf-1 was immunoprecipitated and incubated in 40 μl of a reaction buffer A (40 mM Tris [pH 7.5], 10 mM $MgCl_2$, 30 mM NaCl) containing 50 μM ATP, 30 μCi of [γ-^{3}2P]ATP (3000 Ci/mmol), and either 0.6 mg of kinase-inactive MEK1 (K97M-MKK1; a kind gift of Dr. Natalie Ahn) or 1.25 μg of recombinant human MEK1 (Santa Cruz), 0.625 mg recombinant human ERK1 (UBI), and myelin basic protein (MBP). After 20–30 min, the reaction was stopped by addition of SDS sample buffer. Phosphorylated K97M-MKK1 was resolved by 7.5% SDS–PAGE, and phosphorylated ERK1 and MBP were resolved by 15% SDS–PAGE, prior to autoradiography.

Phosphorylation and Activation of Raf-1 by KSR In Vitro

Immunoprecipitated KSR was incubated with reaction buffer A containing 0.5 mM ATP and a protein kinase inhibitor cocktail (20 μM PKC inhibitor peptide [UBI], 2 μM protein kinase A inhibitor peptide [UBI], and 20 μM R24571 [Sigma]) for 30 min at 22°C. To detect phosphorylation of Raf-1, KSR-bound beads were washed with reaction buffer B and incubated for 30 min with 20 μl of reaction buffer B containing 10 mM $MnCl_2$, 20 μM ATP, 30 μCi of [γ-^{32}P]ATP (3000 Ci/mmol), and recombinant human Raf-1 coexpressed either with Ras and Lck in Sf9 insect cells (UBI) or with recombinant human Flag–Raf-1 immunoprecipitated from COS-7 cells.

To detect activation of Raf-1 in vitro, immunoprecipitated KSR was first incubated with 20 μl of reaction buffer B containing 10 mM $MnCl_2$, 20 μM ATP, and Raf-1 obtained either from Sf9 or COS-7 cells, as above. After 10 min, 20 μl of a reaction mixture containing 30 mM HEPES (pH 7.4), 15 mM $MgCl_2$, 60 mM NaCl, 50 μM ATP, 30 μCi of [γ-^{32}P]ATP (3000 Ci/mmol) was added, and Raf-1 activity was measured as above. It should be noted that when using triply transfected Raf-1 to measure Raf-1 activity in vitro, we first screened each batch for activity by reconstituting signaling through the MAP kinase cascade. Once the activity of each batch of Raf-1 was determined, we reduced the quantity of Raf-1 used in our assays to a level 1/4–1/10 of that used in the screening assay. Routinely, this amounted to 1.2 μl of Raf-1. This rendered the effect of Raf-1 barely detectable and allows for maximal discrimination of the KSR/CAP kinase effect.

Raf Peptide Assay

KSR-bound beads were incubated with reaction buffer B plus 10 mM $MnCl_2$, 5 μM ATP, and [γ-^{32}P]ATP at 22°C in the presence of 0.3 mM synthetic Raf peptide or substrate peptides specific for other Ser/Thr protein kinases. Phosphorylated peptides were resolved by the P81 phosphocellulose paper assay described previously (Mathias et al., 1991). A wild-type Raf-1 peptide derived from the amino acid sequence surrounding Thr^{268} and Thr^{269} of Raf-1 (aa 254–278) contains eight potential phosphorylation sites (shown in bold), RQR**STST**PNVHMV**STT**LPVD**S**RMIE. The amino acid sequences of substrate peptides specific for other protein kinases (Joseph et al., 1993) are: GRGLSLSR (PKA); LSSLRASTSKSGGQK (S6 kinase); RRREEETEEE (CK II); PLARTLSVAGLPGK (CaM kinase II); and VRKRTLRRL (PKC). The Raf-1 peptides and the substrate peptides for S6 kinase, CK II, and CaM kinase were synthesized using an Applied Biosystems model 431A synthesizer. Substrate peptides for PKA and PKC were from Sigma (St. Louis, MO). In some experiments, samples were separated by C18 reverse-phase HPLC (Mathias et al., 1991) to verify the results of the filter assay. Virtually identical results were obtained with both methods.

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