Phosphorylation of the Kinase Suppressor of Ras by Associated Kinases†

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ABSTRACT: The kinase suppressor of Ras (KSR) is a loss-of-function allele that suppresses the rough eye phenotype of activated Ras in *Drosophila* and the multivulval phenotype of activated Ras in *Caenorhabditis elegans*. The physiological role of mammalian KSR is not known. We examined the mechanisms regulating the phosphorylation of this putative kinase in mammalian cells. Wild-type mouse KSR and a mutated KSR protein predicted to create a kinase-dead protein are phosphorylated identically in intact cells and in the immune complex. Phosphopeptide sequencing identified 10 in vivo phosphorylation sites in KSR, all of which reside in the 539 noncatalytic amino terminal amino acids. Expression of the amino terminal portion of KSR alone demonstrated that it was phosphorylated in the intact cell and in an immune complex in a manner indistinguishable from that of intact KSR. These data demonstrate that the kinase domain of KSR is irrelevant to its phosphorylation state and suggest that the phosphorylation of KSR and its association with a distinct set of kinases may affect intracellular signaling.

The small GTPase Ras integrates and transduces extracellular signals through direct protein−protein interactions with downstream effectors (*1*−*4*). One pathway regulated by Ras involves the sequential activation of the cytoplasmic kinases Raf, MEK,[1] and ERK (*5*, *6*). The loss-of-function allele kinase suppressor of Ras encodes a Ras effector recently identified in genetic screens in *Drosophila* and *Caenorhabditis elegans* (*7*−*9*). Epistasis experiments indicated that KSR was a positive regulator of Ras signaling acting in series between Ras and Raf or in parallel to Raf. However, biochemical studies in which the function of KSR was examined indicate that KSR can have both a positive and a negative effect on the ability of Ras to activate ERK MAP kinases and that KSR can exert its effects at different levels of the Raf/MEK/ERK kinase cascade (*10*−*14*). Dissection of the KSR protein has led to the conclusion that its positive effects are mediated by amino acids 319−390 that contain a region of homology (CA3) conserved between KSR proteins of different species (*10*, *11*). The inhibitory effects of KSR on ERK MAP kinase activation have been ascribed to the KSR kinase domain (CA5) (*13*, *14*). The ability of KSR to interact with different components of the Raf/MEK/ERK kinase cascade (*10*−*13*, *15*) has led to the suggestion that KSR may act as a molecular scaffold (*13*).

Despite the fact that the KSR kinase domain can affect ERK MAP kinase activation, the contribution of kinase activity to the biological function of the murine KSR kinase domain is unclear. Unique amino acid substitutions distinguish mammalian KSR kinase domains from their *Drosophila* and *C. elegans* homologues (*7*−*9*). In particular, subdomain II, which contains a lysine in *Drosophila* and *C. elegans* KSR proteins, is substituted with an arginine in mouse and human proteins. This lysine in subdomain II is considered essential for the phosphotransfer reaction and is conserved in all kinases identified previously (*16*). Lysine to arginine mutations at this position are often created experimentally to produce kinase-inactive proteins.

We have observed that KSR is phosphorylated in cells and in an immune complex. In the experiments reported here, we have examined the contribution of the KSR kinase domain to the phosphorylation of KSR. KSR phosphorylation occurred exclusively within the 539 amino acids that precede its carboxyl terminal kinase domain. However, deletion of the KSR kinase domain did not inhibit phosphorylation of KSR in cells or isolated in immune complexes. These data suggest that KSR is phosphorylated by one or more unique kinases that associate with the amino terminal portion of KSR. The identification of 10 sites phosphorylated on KSR in intact cells provides an opportunity to explore the potential of phosphorylation to regulate the biological activity of KSR.

MATERIALS AND METHODS

Cell Culture. The human embryonic kidney cell line 293T (*17*) was maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. All cells were incubated at 37 °C in 5% CO_2.

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[1] Abbreviations: MEK, mitogen- and extracellular-regulated kinase; ERK, extracellular ligand-regulated kinase; KSR, kinase suppressor of Ras; MAP, mitogen-activated protein; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; HPLC, high-pressure liquid chromatography.

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Construction of KSR Expression Plasmids and DNA Mutagenesis. The construction of pCMV5KSRFLAG, KSR C540, and KSR N539 has been reported previously (*14*).

DNA mutagenesis was performed with the QuickChange site-directed mutagenesis kit (Stratagene) according to the manufacturer's directions. Polymerase chain reaction primers were designed to encode changes from serine to alanine or threonine to valine. Where feasible, a silent mutation that created or deleted a restriction endonuclease site was included to facilitate the identification of mutated plasmids. For two sites, the adjacent serines were also mutated to eliminate these as possible alternative phosphorylation sites. The forward primers for mutagenesis were 5′-CCAAGCTGAAGCCACCTAGGGTACCCCCACCGCCAAG-3′ (T274V, creates an *Avr*II site), 5′-CACTCACACGGAGCAAGGCTCATGAGTCCCAGCTGGG-3′ (S297A, creates a *Bsp*HI site), 5′-CTTCGGAGGACAGAGGCGGTACCGTCAGATATCAAC-3′ (S392A, creates a *Kpn*I site), 5′-GAGCCCCATTTTGGAGTGCTTCCCAAGGCCCTG-3′ (T411V, destroys an *Eco*NI site), 5′-GCTGAGGAGCCTGAGGCTGGCAAAGCAGAGGCAGAGGATGACG-3′ (S518A), 5′-GATGCTCGAGACAGTGCCTTGGGGCCTCCCATGGAC-3′ (S190A), 5′-GCCCTGCACAGCTTCATCGTGCCCCCTACCACACCCCAG-3′ (T256V, creates an *Nae*I site), 5′-CCAGCCATGAACCTGGACGCCGCCGCCAACCCATCCTCCACCACG-3′ (S429A/S430A/S431A), 5′-CCCTCATGGAGCCCCACAGCTGGTACGAAGGG-3′ (S320A, destroys the *Bam*HI site), 5′-GCCGCCAACCCAGCCGCCACGACGTCCTCC-3′ (S434A/S435A, creates an *Aat*II site, primer also contains the S430A/S431A mutation), and 5′-GGCGAGGTGGCCATTAAGCTGCTCGAGATGGACGGCCAC-3′ (R589M, creates an *Xho*I site). The reverse primers were the complement of the forward primers. Template DNA was either pCMV5KSR or one of the previously mutated KSR cDNAs. Mutations were confirmed by DNA sequencing.

Transfection, Expression, and Immunoprecipitation of KSR Proteins. Human embryonic kidney 293T cells (5×10^5) were transfected in 35 mm dishes with DNA using $Ca(PO_4)_2$ (*18*). Forty-eight hours after transfection, the cells were frozen on liquid nitrogen and solubilized in buffer A [40 mM Tris-HCl (pH 7.4), 120 mM NaCl, 2 mM EDTA, 2 mM EGTA, 1 mM phenylmethanesulfonyl fluoride, 0.5% Triton X-100, 10 μg/mL leupeptin, 5 μg/mL aprotinin, 10 mM sodium fluoride, and 10 mM sodium pyrophosphate]. Insoluble matter was removed by centrifugation for 10 min at 13000*g* in a microcentrifuge. Cell supernatants (1 mL) were immunoprecipitated for 2 h at 4 °C with 10 μL of anti-FLAG M2 antibodies (Kodak IBI) bound to protein G−agarose, or conjugated directly to agarose. Immunoadsorbed KSR or mutated KSR proteins were washed three times by centrifugation with RIPA buffer [50 mM HEPES (pH 7.8), 0.5% Triton X-100, 1 M NaCl, 0.1% SDS, and 0.1% bovine serum albumin] and once with 50 mM HEPES (pH 7.8) before addition of sample buffer and electrophoresis.

Phosphorylation of KSR Proteins in Intact Cells. 293T cells transiently expressing KSR or the mutated KSR proteins were incubated in phosphate-free Dulbecco's modified Eagle's medium containing [^{32}P]orthophosphate (ICN) at a final concentration of 2 mCi/mL for 4 h at 37 °C, and then lysed in buffer A and immunoprecipitated with anti-FLAG M2 antibodies. Immunoadsorbed KSR or mutated KSR proteins were washed three times by centrifugation with RIPA buffer and once with 50 mM HEPES (pH 7.8) before addition of sample buffer and electrophoresis.

To prepare sufficient KSR protein for protein sequencing, 10 dishes with 293T cells (1.5×10^6 cells/10 cm dish) transiently expressing wild-type KSR or KSR lacking five (KSR 5mut), six (KSR 6mut), or eight (KSR 8mut) identified phosphorylation sites were prepared for each experiment. Two dishes with cells expressing one of the KSR constructs were each labeled with 5 mCi [^{32}P]orthophosphate for 4 h at 37 °C. The remaining dishes were treated in an identical manner but without the addition of radioactivity. Each dish of cells was frozen on liquid nitrogen, lysed in 4 mL of buffer A, and treated as described above with the exception that lysates were incubated in 1.5 mL aliquots at 4 °C for 2 h with 10 μL of anti-FLAG M2 resin, and then incubated overnight at 4 °C with fresh 10 μL aliquots of the anti-FLAG M2 resin. Immunoprecipitates were combined, and KSR was resolved by electrophoresis on an 8% SDS−PAGE gel. KSR proteins were detected with a zinc stain (*19*) and by autoradiography of the wet gel. Trypsin digestion of KSR in situ was performed as described previously (*20*).

Phosphorylation of KSR in the Immune Complex. KSR proteins were immunoprecipitated from 293T cells as described above. Immunoprecipitates were washed three times in buffer A and once in 50 mM HEPES (pH 7.8). Phosphorylation of KSR immunoprecipitates was performed for 1 h at room temperature in a 20 μL reaction volume containing 50 mM HEPES (pH 7.8), 1 mM DTT, 4 mM $MnCl_2$, 10 mM $MgCl_2$, and 5 μM [γ-^{32}P]ATP (50 μCi). Electrophoresis sample buffer was added to stop the reaction. Phosphorylated proteins were resolved by SDS−PAGE and detected by autoradiography or storage phosphor technology (Molecular Dynamics).

Phosphoamino Acid Analysis. Analysis was performed in one or two dimensions on thin layer cellulose plates as described previously (*21*).

HPLC Phosphopeptide Mapping. ^{32}P-labeled KSR proteins were recovered from polyacrylamide gels by the method of Hunter et al. (*22*). The extracted proteins were digested twice with 200 μg/mL (10 μg/50 μL) tosylphenylchloromethyl ketone-treated trypsin in 50 mM ammonium bicarbonate. Phosphopeptides were separated on a Vydac Protein and Peptide C_{18} column with a gradient of 50% acetonitrile in 0.1% trifluoroacetic acid at a flow rate of 1.0 mL/min. Fractions were collected at 1 min intervals and assessed for Cerenkov radiation.

Sequencing of Tryptic Peptide Fragments. Tryptic phosphopeptides generated from radiolabeled KSR were separated on a Vydac C_{18} column (2.1 mm inner diameter × 250 mm) using a Hewlett-Packard 1090 HPLC system with a diode array detector. Peak fractions containing radioactivity were subjected to amino terminal Edman degradation on an Applied Biosystems model 477 protein sequencer. The peptides were attached to Sequelon membranes as described by the manufacturer. The sequencing method for phosphoamino acids (*23*) was modified to allow radioactivity from phosphoserine and phosphothreonine to be observed in the collected fractions. The Sequelon membrane was dried and washed sequentially with methanol, water, methanol, water, and 10% trifluoroacetic acid/50% acetonitrile for 5 min each. The S3 position on the sequencer was changed to 0.015%

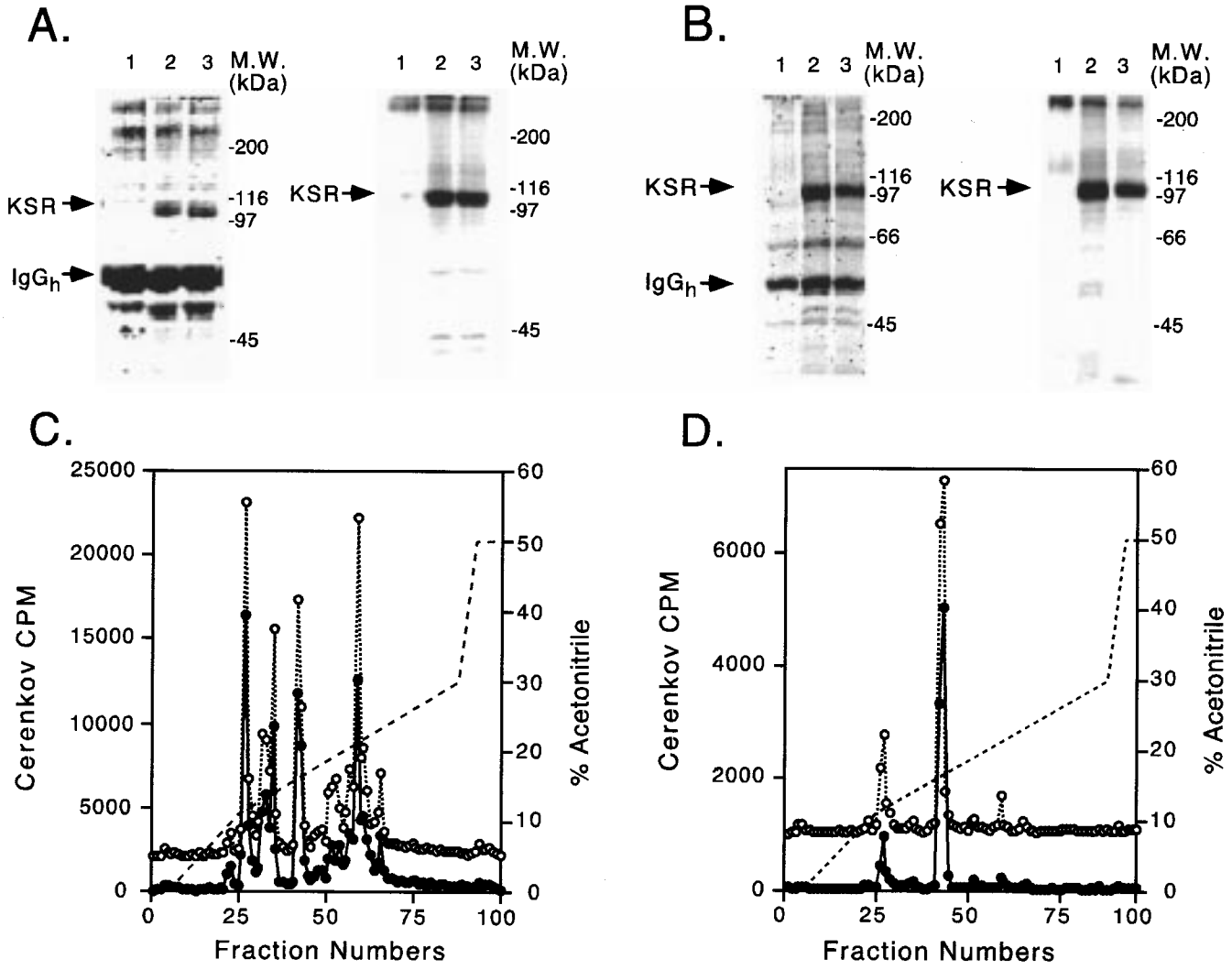

FIGURE 1: Phosphorylation of KSR in cells. (A) 293T cells transfected with pCMV5 alone (lane 1), pCMV5KSR (lane 2), or pCMV5KSR R589M (lane 3) were labeled with ^{32}P, lysed, and immunoprecipitated with an antibody to the FLAG epitope tag. Coomassie-stained proteins immunoprecipitated from each lysate (left panel) and an autoradiograph of the gel (right panel) are shown. Arrows denote the position of KSR proteins. (B) Immunoprecipitates were made from unlabeled 293T cells transfected as described for panel A. Precipitated proteins were phosphorylated in the immune complex with [^{32}P]ATP and separated by electrophoresis. Coomassie-stained proteins immunoprecipitated from each lysate (left panel) and an autoradiograph of the gel (right panel) are shown. Arrows denote the position of KSR proteins. The numbering of the lanes is the same as in panel A. (C) Tryptic phosphopeptides from ^{32}P-labeled KSR (○, offset by 2000 cpm) and KSR R589M (●) in the panel A gel were analyzed by HPLC. The acetonitrile gradient is shown by the dashed line. (D) Tryptic phosphopeptides from ^{32}P-labeled KSR (○, offset by 1000 cpm) and KSR R589M (●) in the panel B gel were analyzed by HPLC.

phosphoric acid/90% methanol. The programming for the sequencer was modified to include three transfer steps of 30 s each using phosphoric acid/methanol. Heptane was delivered to the midpoint of the cartridge block followed by a 10 s pause prior to each transfer. For solubilization of the phenylthiohydantoin derivative, S4 (20% acetonitrile) was loaded four times for a total volume of 300 μL. Fifty microliters of sample was injected onto the attached ABI 120 HPLC system, while the remainder of the sample was shunted to a fraction collector. The internal restrictor line of the HPLC was altered, and the final step of sequencing each radioactive peptide was changed to a cleaning cycle to avoid blockage of the injection valve. No phenylthiohydantoin derivative was seen on the chromatograph for these phosphorylated amino acids; however, the remainder of the peptide sequence could be determined. The average repetitive yield of the sequencer when this protocol was used was determined to be 90% when measured with a standard.

Renaturation of Kinase Activity. Cells expressing KSR or the mutated KSR proteins were frozen on liquid nitrogen and lysed in buffer A without sodium fluoride and sodium pyrophosphate. The lysate was clarified at 13000*g* for 10 min to pellet the debris and immunoprecipitated with 20 μL of anti-FLAG M2 resin for 2 h. Samples were washed by centrifugation three times with 1 mL of RIPA buffer and once with 1 mL of 50 mM HEPES (pH 7.8). Electrophoresis sample buffer containing 5% 2-mercaptoethanol was added, and the samples were heated at 110 °C for 3 min. Samples were separated on 8% polyacrylamide gels polymerized in the presence of 0.5 mg/mL myelin basic protein. Each gel was renatured and assayed for kinase activity as described previously (*24*) except that the assay buffer was identical to that used in the immune complex kinase assay. Phosphorylated proteins were detected by autoradiography or storage phosphor technology (Molecular Dynamics).

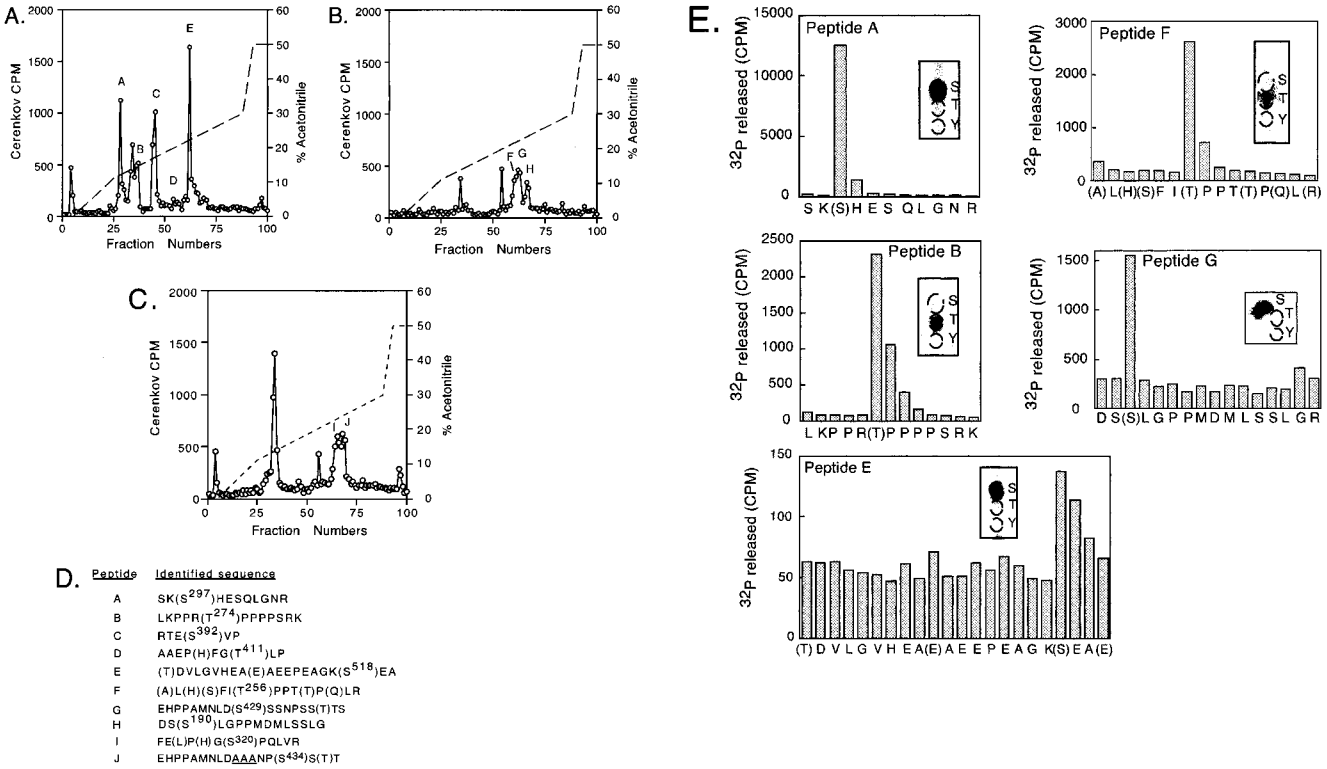

FIGURE 2: Identification of the phosphorylation sites of KSR by radiosequencing. Radiolabeled phosphopeptides were generated by trypsin digestion of intact and mutated ^{32}P-labeled KSR proteins that were immunoprecipitated from 293T cells transfected with pCMV5KSR. (A) Phosphopeptides were separated by HPLC and detected by counting the samples for Cerenkov radiation. A typical separation profile is shown. (B) A second HPLC phosphopeptide map was generated from a mutated KSR protein lacking the first five phosphorylation sites identified in panel A. (C) Additional phosphopeptides were identified in peak G from a mutated KSR protein lacking the eight phosphorylation sites identified in panels A and B. Peaks containing phosphopeptides isolated and sequenced from the profiles in panels A−C are denoted by capital letters. (D) The sequence determined for each of the isolated phosphopeptides is listed. Amino acids listed in parentheses were deduced from the known sequence of KSR. Underlined amino acids represent phosphorylation sites mutated prior to analysis and radiosequencing. (E) The release of ^{32}P was assessed in each sequencing cycle of phosphopeptides in peaks A, B, and E−G. The sequence of the identified peptides is reproduced at the bottom of each graph. Phosphoamino acid analysis of a portion of each sequenced phosphopeptide is shown in the insets. The location of phosphoamino acid standards is denoted. S is serine. T is threonine. Y is tyrosine.

Western Blot Analysis. Proteins were transferred to polyvinylidene difluoride membranes, and Western blots were developed using anti-FLAG M2 antibodies (1:3000). Blots were developed with 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium using goat anti-mouse secondary antibody conjugated to alkaline phosphatase.

RESULTS

Mouse KSR Is Phosphorylated in Intact Cells and in the Immune Complex. Mammalian KSR proteins contain five regions conserved in homologues from *Drosophila* and *C. elegans*. The largest region of sequence identity between KSR proteins of different species is the putative kinase domain (*7*). One striking difference between mammalian KSR and KSR from *Drosophila* and *C. elegans* resides within subdomain II of the protein kinase domain. Subdomain II in protein kinases contains an invariant lysine that is required for transferring phosphate from ATP to substrate (*16*). This lysine is conserved in the kinase domains of KSR from *Drosophila* and *C. elegans* but is replaced with an arginine in the mammalian KSR proteins. Arginine substitutions at this position typically inactivate protein kinases (*25*) and raise the possibility that mammalian KSR proteins might not retain catalytic activity. The extent of phosphorylation of KSR and KSR R589M, a mutation which should render the kinase domain of KSR fully inactive, was measured. Both KSR and KSR R589M proteins were phosphorylated to the same extent in intact cells (Figure 1A) or in an immune complex (Figure 1B), suggesting that the kinase domain may not contribute to KSR phosphorylation. Phosphorylated KSR proteins were eluted from the gel and digested with trypsin, and the radiolabeled phosphopeptides were separated by HPLC. Tryptic phosphopeptides generated from KSR and KSR R589M created the same phosphopeptide map both in intact cells (Figure 1C) and when phosphorylated in an immune complex (Figure 1D). The reduced intensity of ^{32}P-labeled phosphopeptides derived from KSR phosphorylated in the immune complex may be due to the presence of preexisting, endogenous phosphate on KSR proteins isolated from cells in the presence of phosphatase inhibitors.

Identification of KSR Phosphorylation Sites. Ten sites of KSR phosphorylation were determined by peptide sequence analysis as an initial step toward testing the potential of phosphorylation to regulate KSR function. The first round of sequencing identified five of the major sites of phosphorylation. The elution positions of the phosphopeptides containing these sites are shown in Figure 2A. Confirmation of the phosphorylation of these sites is shown by the disappearance of those five peaks in the phosphorylation profile of a mutated KSR protein in which serines were changed to alanines and threonines were changed to valines at these five sites (Figure 2B). The mutation of these five

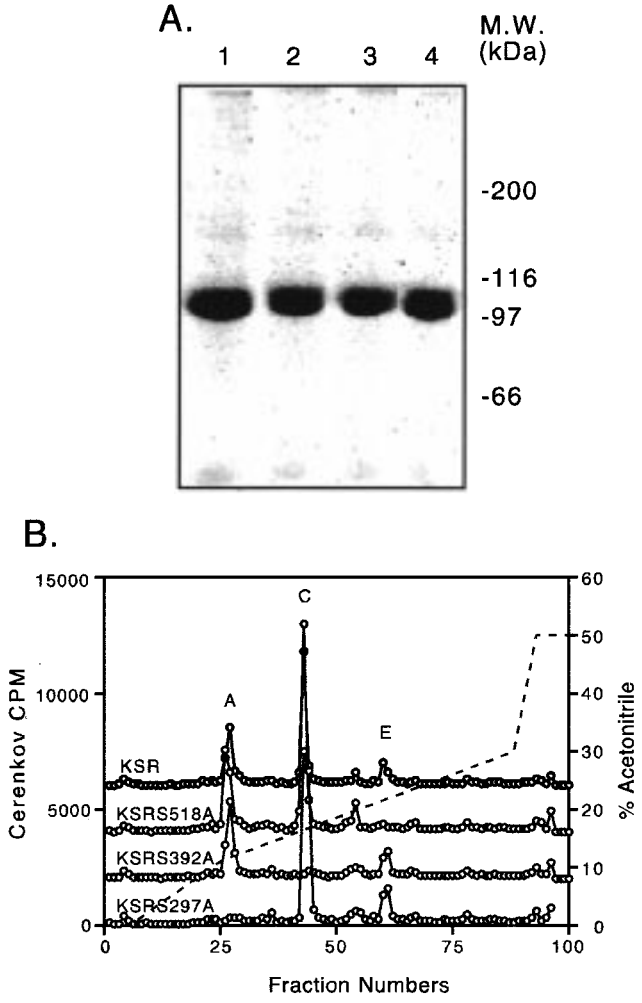

FIGURE 3: Phosphorylation of intact and mutated KSR proteins in the immune complex. (A) Lysates from 293T cells transiently expressing intact KSR (lane 1), KSR S297A (lane 2), KSR S392A (lane 3), and KSR S518A (lane 4) were immunoprecipitated with anti-FLAG antibodies, and each immune complex was incubated with [^{32}P]ATP. Phosphorylated proteins were resolved by electrophoresis and autoradiography. (B) The HPLC analysis of the phosphopeptides derived from each protein is shown. Peptides A, C, and E correspond to the sequences identified in Figure 2. Samples are offset by 2000 cpm for clarity.

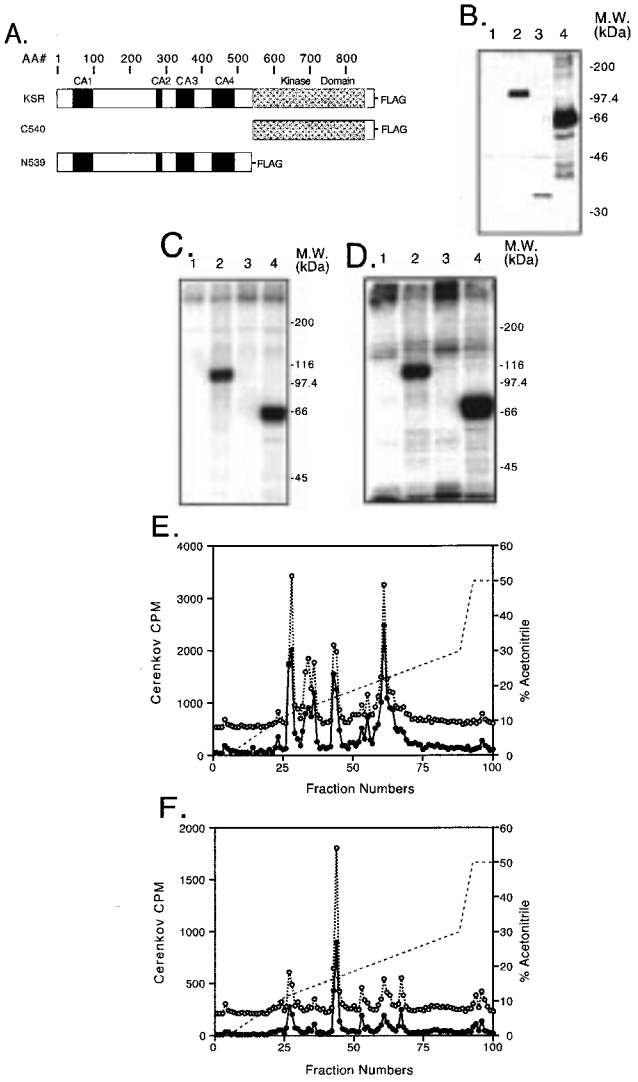

FIGURE 4: Phosphorylation of KSR, KSR N539, and KSR C540 in 293T cells and in the immune complex. (A) Schematic representation of KSR and truncated KSR constructs KSR C540 and KSR N539. (B) Western blot of immunoprecipitated KSR proteins. 293T cells were transfected with pCMV5 alone (lane 1), pCMV5KSR (lane 2), pCMV5KSR C540 (lane 3), or pCMV5KSR N539 (lane 4), lysed, immunoprecipitated, and probed with anti-FLAG antibodies. (C) 293T cells transfected as described for panel A were labeled with ^{32}P. KSR proteins were immunoprecipitated and resolved by SDS−PAGE, and the gel was subjected to autoradiography. Lane assignments are identical to those in panel B. (D) Immune complex kinase assays with [^{32}P]ATP were performed on anti-FLAG immunoprecipitates from control 293T cells (lane 1) or cells expressing KSR (lane 2), KSR C540 (lane 3), and KSR N539 (lane 4). ^{32}P-labeled proteins were resolved by SDS−PAGE and identified by autoradiography. (E) HPLC analysis of phosphopeptides derived from KSR (○, offset by 500 cpm) and KSR N539 (●) samples shown in panel C. (F) HPLC analysis of phosphopeptides derived from KSR (○, offset by 200 cpm) and KSR N539 (●) samples shown in panel D.

sites leads to a relative increase in the degree of phosphorylation of other, minor sites. Five additional sites were identified in subsequent experiments. Mutation of these sites was responsible for the loss of additional peaks as demonstrated in Figure 2C. Phosphorylation of Ser^{434} (peptide I, Figure 2D) was not detected until the phosphorylation site Ser^{429} was mutated. This observation suggests that Ser^{434} may be an alternative phosphorylation site in KSR. Figure 2D shows the sequence determined by Edman degradation of the KSR phosphopeptides identified in panels A and B of Figure 2. The amount of ^{32}P released in each cycle by Edman degradation of each phosphopeptide corresponded precisely with the predicted location of each phosphorylation site (Figure 2E). In addition, phosphoamino acid analysis of each phosphopeptide peak corresponded to the prediction of phosphoserine or phosphothreonine in each phosphopeptide that was sequenced (insets of Figure 2E).

Phosphorylation of Mutated KSR Proteins in an Immune Complex. Mutated KSR proteins lacking predicted in vivo phosphorylation sites at Ser^{297}, Ser^{392}, and Ser^{518} were tested for their ability to be phosphorylated in an immune complex (panels A and B of Figure 3). Phosphorylated KSR S297A, KSR S392A, and KSR S518A each exhibit a specific loss of one radioactive peak at the expected elution position for the peptide containing that phosphorylation site. Ser^{392} is the major phosphorylated peak in a KSR immune complex, possibly due to the fact that this site is phosphorylated to a lower stoichiometry in the intact cell. Thus, Ser^{392} could be ^{32}P-labeled in the immune complex to a higher stoichiometry.

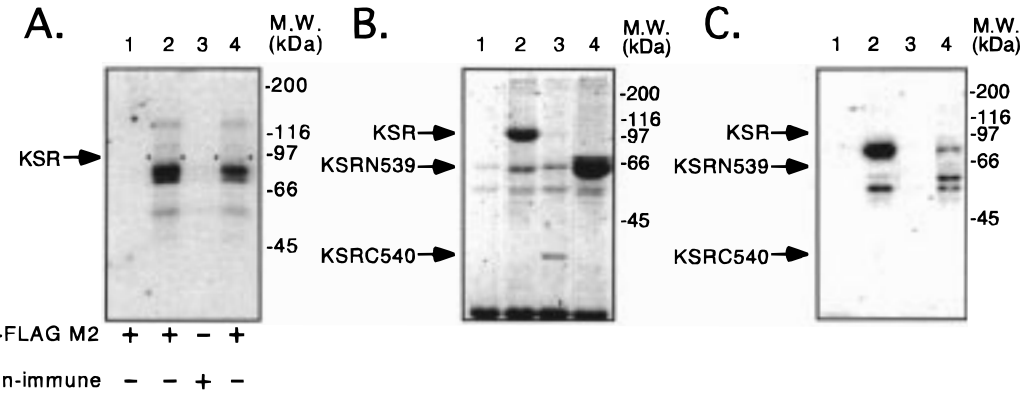

FIGURE 5: Renaturation of kinase activity in KSR immunoprecipitates. (A) Lysates from 293T cells transfected with the expression vector alone (lane 1), expressing KSR (lanes 2 and 3), or expressing KSR R589M (lane 4) were immunoprecipitated with anti-FLAG or nonimmune antibodies as indicated. The immunoprecipitates were separated by electrophoresis on an 8% SDS−PAGE gel, and the proteins were renatured in the gel. Renatured kinase activity within the gel was detected with the addition of [^{32}P]ATP, and the position of labeled bands was determined by autoradiography. The relative position of Coomassie-stained KSR proteins in the renatured gel is denoted by the arrow and the dots adjacent to lanes 2 and 4. (B and C) Lysates from 293T cells transfected with the expression vector alone (lane 1), expressing KSR (lane 2), expressing KSR C540, or expressing KSR N539 were immunoprecipitated with anti-FLAG M2 antibody. The immunoprecipitates were separated by electrophoresis on a 10% SDS−PAGE gel, and the proteins were renatured in the gel. Renatured kinase activity was detected in the gel as described for panel A. The proteins immunoprecipitated from each lysate (B) were stained with Coomassie, and the kinase activity present in each lane (C) was identified by autoradiography. Arrows denote the position of the different KSR proteins.

In contrast, in the intact cell, Thr^{274}, Ser^{297}, Ser^{392}, and Ser^{518} appear to phosphorylate equally well.

Phosphorylation of KSR Deletion Mutants. The inability of the Met^{589} mutation to affect the phosphorylation of KSR in intact cells or in an immune complex suggested the possibility that KSR is not autophosphorylated but phosphorylated by associated kinases. The in vivo phosphorylation sites that were identified (Figure 2) were all located amino terminal to the KSR kinase domain. Truncated constructs of KSR cDNA (Figure 4A) were created that expressed the KSR kinase domain alone (KSR C540) or only sequences amino terminal to the KSR kinase domain (KSR N539). The FLAG epitope tag was added to the carboxyl terminus of both truncated proteins. Each truncated, recombinant protein was compared to intact, full-length KSR for its ability to be phosphorylated in intact human embryonic kidney 293T cells (Figure 4C), or after immunoprecipitation from these cells (Figure 4D). No detectable phosphorylation of KSR C540 was detected in intact cells or in an immune complex (panels C and D of Figure 4). These observations are consistent with the observation that phosphorylation sites on KSR are confined to structures amino terminal to the kinase domain. In contrast to the KSR kinase domain, KSR N539 was phosphorylated strongly in intact cells and in the immune complex (panels B and C of Figure 4). This observation was surprising since KSR N539 does not contain the KSR kinase domain. HPLC mapping revealed that intact KSR and KSR N539 are phosphorylated on the same sites in intact cells and in an immune complex (panels E and F of Figure 4).

Renaturation of Kinase Activity in KSR Immunoprecipitates. The observation that the noncatalytic KSR N539 was phosphorylated in an immune complex on the same sites phosphorylated on intact KSR in cells suggests that the amino terminal portion of KSR is not phosphorylated by the KSR kinase domain. These data further suggest that the kinase that does phosphorylate KSR in intact cells binds to and coprecipitates with its amino terminal portion. To test this possibility, immunoprecipitates of intact KSR, KSR R589M, KSR C540, and KSR N539 were subjected to an "in gel" kinase renaturation assay. Renaturation of wild-type KSR or KSR R589M demonstrated the presence of several coprecipitating and renatured kinases that were absent when mock transfected 293T cells were immunoprecipitated with the anti-FLAG antibody, or when 293T cells transfected with pCMV5KSRFLAG were immunoprecipitated with a nonspecific antibody (Figure 5A). The most prominent renatured kinase migrated on SDS−PAGE slightly faster than the KSR proteins that were expressed in 293T cells and detected in the gel by Coomassie blue staining. The renatured kinases coprecipitating with KSR also precipitated with the amino terminal construct KSR N539, but with altered stoichiometries (Figure 5C). No renatured kinase activity was detected in immunoprecipitates of the KSR kinase construct C540.

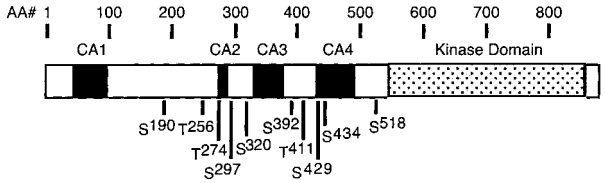

FIGURE 6: Schematic of KSR and the KSR mutants. A schematic of mouse KSR indicating the areas (CA) conserved between mouse KSR and KSR proteins from different species. The location of identified phosphorylation sites is denoted.

DISCUSSION

The data presented here demonstrate that murine KSR is phosphorylated in cells and in an immune complex on at least 10 distinct serine and threonines. Phosphorylation is confined to the amino terminal portion of KSR, excluding the kinase domain (Figure 6). The KSR kinase domain does not contribute to phosphorylation because deletion of the kinase domain does not prevent the phosphorylation of the isolated amino terminal portion of KSR (KSR N539). These data suggest, instead, that one or more distinct kinases associate with and phosphorylate the KSR amino terminal region. The observation of the fact that KSR is phosphory-

lated in an immune complex on the same sites that are phosphorylated on KSR in intact cells suggests that the associated kinase that phosphorylates KSR may be physiologically relevant to KSR function.

No consensus sequence can be applied to all of the phosphorylation sites that have been identified. However, six of the phosphorylated amino acids (Ser^{190}, Thr^{256}, Thr^{274}, Ser^{320}, Ser^{392}, and Thr^{411}) are followed by a proline at position P + 1, P + 2, or P + 3. The sequences adjacent to two other phosphorylation sites that were identified (Ser^{297} and Ser^{518}) appear to be unrelated but are both notable for having a lysine at position P − 1. The other two phosphorylation sites that were identified (Ser^{429} and Ser^{434}) are located in the highly serine/threonine rich CA4 region. One explanation for the lack of sequence specificity between the identified phosphorylation sites is the possibility that more than one kinase binds to the amino terminal region of KSR and each kinase phosphorylates different sites.

The potential of KSR to interact with sequential members of a signaling cascade has led to the suggestion that KSR may serve as a scaffold protein (*13*). Scaffold proteins simultaneously associate with several effectors of a signaling pathway to create an ordered module that facilitates sequential activation of the associated enzymes (*26*). In *Saccharomyces cerevisiae*, Ste5p is a scaffold protein that binds yeast homologues of the mammalian ERK MAP kinase cascade (*27*). KSR is unrelated to Ste5p, but through coprecipitation or two-hybrid analysis, KSR has been reported to interact with Raf, MEK, and ERK MAP kinases (*10*, *12*, *13*, *15*). MEK (*12*, *13*) and Raf (*10*) have been observed to interact with the carboxyl terminal kinase domain of KSR, whereas ERK MAP kinase has been reported to interact with sequences in KSR amino terminal to the kinase domain (*13*, *28*). Only one of the KSR phosphorylation sites identified here (Thr^{274}) has the consensus sequence for ERK MAP kinase phosphorylation (P-X-S/T-P) (*29*, *30*). Thus, it is possible that ERK MAP kinases are responsible, at least in part, for the phosphorylation of KSR. In addition to the phosphorylation of Thr^{274}, ERK MAP kinases have also been suggested to be responsible for the phosphorylation of Thr^{260} and Ser^{443} in KSR (*13*, *31*). However, the phosphorylation of other sites cannot be ascribed to the activity of ERK MAP kinases (*31*). The association of ERK MAP kinases with KSR in mammalian cells is dependent on their activation (*31*). We did not detect ERK MAP kinases in immunoprecipitates of KSR (data not shown). We have observed that the expression of KSR in 293T cells inhibits the activation of ERK MAP kinases (*14*), which may explain our inability to detect their association with KSR. The association of Raf-1 and MEK with KSR has also been detected. However, the association of Raf-1 and MEK with KSR is mediated through the KSR kinase domain (*10*, *12*, *13*). The observation that KSR phosphorylation can be recapitulated in immunoprecipitates of the KSR N539 construct that lacks the kinase domain suggests that Raf-1 and MEK are not the kinases responsible for the phosphorylation of KSR. We also detected no renatured kinase activity associated with the KSR kinase domain (Figure 5), and the renatured kinases associated with KSR N539 did not have the electrophoretic mobility of p42/p44 ERK MAP kinases.

These observations suggest that KSR may have the ability to bind multiple kinases as a mechanism for regulating intracellular signaling. Still unanswered by the studies reported here and by other laboratories is the question of whether each molecule of KSR is capable of simultaneously binding all of the known and uncharacterized proteins with which it interacts. It is possible that some of the kinases coprecipitating with KSR contain homologous structures that permit each of them to bind to the same region of KSR. It is also possible that phosphorylation of KSR regulates the binding of KSR to other proteins either in the KSR kinase domain or to sequences amino terminal to the kinase domain. The identification of in vivo phosphorylation sites makes those experiments feasible.

A positive effect on the Ras/Raf/MEK/ERK MAP kinase signaling cascade has been ascribed to the amino terminal portion of KSR (*10*, *11*), while inhibition of the Ras/Raf/MEK/ERK MAP kinase signaling cascade has been localized to the carboxyl terminal kinase domain (*12*−*14*). The stimulatory effects of KSR on Ras signaling have been localized to amino acids 319−390, which contain the cystine rich CA3 region of KSR (*11*). The sequence of KSR CA3 is homologous with those of atypical C1 domain-containing proteins which include c-Raf-1 (*32*). C1 domains are believed to be involved in the membrane localization of the proteins that contain them. Localization of KSR to a particular cell fraction is dependent on its CA3 domain (*11*, *15*), and this capability is necessary but not sufficient for the stimulatory actions of KSR on Raf (*11*). None of the phosphorylation sites identified in this study reside within the CA3 region of KSR. However, Ser^{392}, immediately adjacent to the KSR CA3 domain, is phosphorylated (Figure 2) and has been suggested as a site for binding of 14-3-3 protein to KSR (*15*, *31*). The proximity of Ser^{392} to the CA3 region of KSR makes it a potential regulator of KSR subcellular localization and KSR-mediated effects on Ras signaling.

Renaturation of intact KSR in polyacrylamide gels led others to conclude that KSR was autophosphorylated (*33*). The data shown here demonstrate that phosphorylation of KSR is not autocatalytic, but is instead catalyzed by a kinase associated with the 539 noncatalytic, amino terminal amino acids of KSR. Renaturation of a KSR-associated kinase with electrophoretic mobility nearly identical to but slightly faster than that of KSR (Figure 5) may explain, in part, the difference in the interpretation of the results described previously (*33*) and the data presented here. However, the fact that the same kinase activity binds to the truncated, noncatalytic KSR N539 construct demonstrates the difference between intact KSR and coprecipitating kinases (Figure 5). It is possible that the complete phosphorylation of KSR is dependent on association with one or more of the unidentified kinases that bind KSR. Molecular dissection of KSR will permit an analysis of this hypothesis and should be helpful in identifying a physiological role for phosphorylation in the control of KSR signal transduction.

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REFERENCES

1. Schlessinger, J., and Bar-Sagi, D. (1994) *Cold Spring Harbor Symp. Quant. Biol. 59*, 173−180.

2. White, M. A., Nicolette, C., Minden, A., Polverino, A., Van Aelst, L., Karin, M., and Wigler, M. H. (1995) *Cell 80*, 533−541.
3. Rodriguez-Viciana, P., Warne, P. H., Dhand, R., Vanhaesebroeck, B., Gout, I., Fry, M. J., Waterfield, M. D., and Downward, J. (1994) *Nature 370*, 527−532.
4. Van Aelst, L., Barr, M., Marcus, S., Polverino, A., and Wigler, M. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 6213−6217.
5. Marshall, C. J. (1996) *Curr. Opin. Cell Biol. 8*, 197−204.
6. Khosravi-Far, R., and Der, C. J. (1994) *Cancer Metastasis Rev. 13*, 67−89.
7. Therrien, M., Chang, H. C., Solomon, N. M., Karim, F. D., Wasserman, D. A., and Rubin, G. M. (1995) *Cell 83*, 879−888.
8. Sundaram, M., and Han, M. (1995) *Cell 83*, 889−901.
9. Kornfeld, K., Hom, D. B., and Horvitz, H. R. (1995) *Cell 83*, 903−913.
10. Therrien, M., Michaud, N. R., Rubin, G. M., and Morrison, D. K. (1996) *Genes Dev. 10*, 2684−2695.
11. Michaud, N. R., Therrien, M., Cacace, A., Edsall, L. C., Spiegel, S., Rubin, G. M., and Morrison, D. K. (1997) *Proc. Natl. Acad. Sci. U.S.A. 94*, 12792−12796.
12. Denouel-Galy, A., Douville, E. M., Warne, P. H., Papin, C., Laugier, D., Calothy, G., Downward, J., and Eychène, A. (1998) *Curr. Biol. 8*, 46−55.
13. Yu, W., Fantl, W. J., Harrowe, G., and Williams, L. T. (1998) *Curr. Biol. 8*, 56−64.
14. Joneson, T., Fulton, J. A., Volle, D. J., Chaika, O. V., Bar-Sagi, D., and Lewis, R. E. (1998) *J. Biol. Chem. 273*, 7743−7748.
15. Xing, H. M., Kornfeld, K., and Muslin, A. J. (1997) *Curr. Biol. 7*, 294−300.
16. Hanks, S. K., and Hunter, T. (1995) *FASEB J. 9*, 576−596.
17. Pear, W. S., Nolan, G. P., Scott, M. L., and Baltimore, D. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 8392−8396.
18. Rogers, J. A., Read, R. D., Li, J. Z., Peters, K. L., and Smithgall, T. E. (1996) *J. Biol. Chem. 271*, 17519−17525.
19. Fernandez-Patron, C., Castellanos-Serra, L., and Rodriguez, P. (1992) *BioTechniques 12*, 564−573.
20. Rosenfeld, J., Capdevielle, J., Guillemot, J. C., and Ferrara, P. (1992) *Anal. Biochem. 203*, 173−179.
21. Tauer, T. J., Volle, D. J., Rhode, S. L., and Lewis, R. E. (1996) *J. Biol. Chem. 271*, 331−336.
22. Boyle, W. J., Van der Geer, P., and Hunter, T. (1991) *Methods Enzymol. 201*, 110−149.
23. Wettenhall, R. E., Aebersold, R. H., and Hood, L. E. (1991) *Methods Enzymol. 201*, 186−199.
24. Kameshita, I., and Fujisawa, H. (1989) *Anal. Biochem. 183*, 139−143.
25. Hanks, S. K., Quinn, A. M., and Hunter, T. (1988) *Science 241*, 42−52.
26. Faux, M. C., and Scott, J. D. (1996) *Cell 85*, 9−12.
27. Marcus, S., Polverino, A., Barr, M., and Wigler, M. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 7762−7766.
28. Jacobs, D., Glossip, D., Xing, H., Muslin, A. J., and Kornfeld, K. (1999) *Genes Dev. 13*, 163−175.
29. Alvarez, E., Northwood, I. C., Gonzalez, F. A., Latour, D. A., Seth, A., Abate, C., Curran, T., and Davis, R. J. (1991) *J. Biol. Chem. 266*, 15277−15285.
30. Gonzalez, F. A., Raden, D. L., and Davis, R. J. (1991) *J. Biol. Chem. 266*, 22159−22163.
31. Cacace, A. M., Michaud, N. R., Therrien, M., Mathes, K., Copeland, T., Rubin, G. M., and Morrison, D. K. (1999) *Mol. Cell. Biol. 19*, 229−240.
32. Hurley, J. H., Newton, A. C., Parker, P. J., Blumberg, P. M., and Nishizuka, Y. (1997) *Protein Sci. 6*, 477−480.
33. Zhang, Y., Yao, B., Delikat, S., Bayoumy, S., Lin, X.-H., Basu, S., McGinley, M., Chan-Hui, P.-Y., Lichenstein, H., and Kolesnick, R. (1997) *Cell 89*, 63−72.

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