Published in final edited form as:
Mol Cell. 2009 June 26; 34(6): 652–662. doi:10.1016/j.molcel.2009.06.00.

KSR2 is a Calcineurin Substrate that Promotes ERK Cascade Activation in Response to Calcium Signals

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SUMMARY

Protein scaffolds have emerged as important regulators of MAPK cascades, facilitating kinase activation and providing crucial spatio/temporal control to their signaling outputs. Using a proteomics approach to compare the binding partners of the two mammalian KSR scaffolds, we find that both KSR1 and KSR2 interact with the kinase components of the ERK cascade and have a common function in promoting RTK-mediated ERK signaling. Strikingly, we find that the protein phosphatase calcineurin selectively interacts with KSR2 and that KSR2 uniquely contributes to Ca^{2+}-mediated ERK signaling. Calcineurin dephosphorylates KSR2 on specific sites in response to Ca^{2+} signals, thus regulating KSR2 localization and activity. Moreover, we find that depletion of endogenous KSR2 impairs Ca^{2+}-mediated ERK activation and ERK-dependent signaling responses in INS1 pancreatic β-cells and NG108 neuroblastoma cells. These findings identify KSR2 as a Ca^{2+}-regulated ERK scaffold and reveal a new mechanism whereby Ca^{2+} impacts Ras to ERK pathway signaling.

INTRODUCTION

Signal transduction is a complex process whereby cells translate extracellular signals into specific biological responses. In many cell types, modules of sequentially activating protein kinases, such as the MAPK cascades, are essential for this process (Pearson et al., 2001; Qi and Elion, 2005). These kinase cascades function as integrators of signaling inputs and serve as relay routes from the cell surface to the nucleus. One signaling pathway that plays a critical role in the transmission of many growth and developmental cues is the pathway regulated by the Ras GTPase (Malumbres and Barbacid, 2003). In higher eukaryotes, an essential kinase module used in Ras-dependent signaling is the MAPK cascade comprised of the Raf/MEK/ERK kinases (Marshall, 1996).

Although the kinase components of the ERK cascade can interact via a series of sequential binary interactions, these kinases can also be organized into complexes by scaffold proteins. The importance of scaffolds to MAPK signaling originates from studies of the Ste5p protein

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SUPPLEMENTAL DATA

The Supplemental Data include Supplemental Experimental Procedures, four figures and one table.

in budding yeast, where Ste5p functions as an essential docking platform for MAPK components during pheromone-induced mating (Elion, 2001). Scaffold proteins facilitate kinase activation by colocalizing the core kinase components of the cascade and they provide spatial regulation through their localization to specific subcellular sites (Dard and Peter, 2006; Shaul and Seger, 2007). In addition, scaffolds can impact the duration and amplitude of signaling by interacting with positive and negative regulators of the cascade (Hao et al., 2008).

An important scaffold for ERK cascade signaling is the Kinase Suppressor of Ras (KSR) family (Kolch, 2005; Morrison and Davis, 2003). The KSR proteins were first identified as positive regulators of Ras pathway signaling through genetic studies performed in *Drosophila melanogaster* and *Caenorhabditis elegans* (Kornfeld et al., 1995; Sundaram and Han, 1995; Therrien et al., 1995). Subsequent biochemical analysis revealed that members of the KSR family interact with the core kinase components of the ERK cascade to facilitate Ras to ERK signaling at the plasma membrane (Denouel-Galy et al., 1998; Muller et al., 2001; Roy et al., 2002; Therrien et al., 1996). One KSR protein is present in *Drosophila*, whereas two KSR family members are found in *C. elegans*, invertebrates, and mammals. In flies, a functional KSR protein is required for viability (Therrien et al., 1995), and, in worms, the two KSR proteins are needed for most aspects of Ras-mediated signaling (Ohmachi et al., 2002; Sundaram et al., 1996). Specifically, *ksr-1* is required for sex myoblast migration and is a nonessential positive regulator of Ras-mediated developmental events, whereas *ksr*-2 is required for Ras-mediated germline meiotic progression and functions redundantly with *ksr-1* during worm development.

The best characterized of the mammalian KSR proteins is KSR1. Murine KSR1 interacts constitutively with MEK and translocates to the cell surface following RTK-mediated Ras activation, thus colocalizing MEK with Raf at the plasma membrane (Muller et al., 2001). In addition, KSR1 has been reported to associate with various signaling modulators, including the positive regulators protein phosphatase 2A (PP2A; Ory et al., 2003) and casein kinase 2 (CK2; Ritt et al., 2007), and the negative regulators 14-3-3 (Cacace et al., 1999; Xing et al., 1997), the C-TAK1 kinase (Muller et al., 2001), and the E3 ubiquitin ligase IMP (Matheny et al., 2004). KSR1 knock-out mice have also been generated. These animals are grossly normal but display subtle defects in T-cell activation and certain types of neuronal signaling (Nguyen et al., 2002; Shalin et al., 2006), suggesting that there may be significant functional overlap between KSR1 and KSR2. To date, only limited characterization of a truncated human KSR2 protein has been reported. In these studies, a KSR2 construct lacking the first two coding exons of human KSR2 was found to inhibit signaling downstream of the MEKK3 and Cot/Tpl2 kinases (Channavajhala et al., 2005; Channavajhala et al., 2003).

To further characterize KSR2 and to identify functionally important differences in the murine KSR scaffolds, we performed a proteomics analysis of the KSR1 and KSR2 scaffold complexes. Here, we report that KSR2, like KSR1, interacts with components of the ERK cascade and facilitates ERK cascade activation in a concentration-dependent manner. Notably, we find that the protein phosphatase calcineurin selectively interacts with KSR2 and regulates KSR2 localization and ERK scaffold activity in response to Ca^{2+} signals. Moreover, depletion of KSR2 is found to impair Ca^{2+}-mediated ERK activation and signaling in two different cell lines that express KSR2, INS1 pancreatic β-cells and NG108 neuroblastoma cells. These findings identify a distinct function for KSR2 in Ca^{2+}-regulated ERK signaling.

RESULTS

Common Properties of KSR1 and KSR2

Murine KSR2 is a 945 amino acid protein that shares 43% sequence identity (54% similarity) with murine KSR1 and possesses all the conserved domains (CA1-CA5) previously identified in KSR family members (Therrien et al., 1995). In addition, KSR2 contains residues analogous to those in KSR1 that are known to be required for binding interactions with MEK, ERK, Raf, and the 14-3-3 proteins (Figure 1A). Unique to KSR2 is the presence of a 63 amino acid region located between the CA2 and CA3 domains.

The KSR1 scaffold is known to exist as a large multi-protein complex (Ory et al., 2003; Stewart et al., 1999), and given that many of the molecules involved in the ERK scaffold function of KSR1 are associated proteins, we reasoned that insights into KSR2's function might be gained by identifying KSR2 binding partners. Moreover, a comparison of the protein constituents of the KSR1 and KSR2 complexes might reveal unique functions and regulatory mechanisms for each of the KSR scaffolds. Therefore, we isolated KSR1 and KSR2 complexes from cycling 293T cells and determined the proteins present in these complexes using mass spectrometry (Figures 1B and S1, and Table S1). As verification of this approach, many of the known KSR1-binding partners were detected in KSR1 complexes and were also present in KSR2 complexes (Figure 1B). These proteins include the ERK cascade components B-Raf, MEK and ERK; the chaperone proteins Hsp90 and cdc37; and proteins involved in regulating KSR1 localization, such as C-TAK1, 14-3-3, and PP2A. The identification of these proteins in KSR2 complexes suggests that KSR2, like KSR1, may function as an ERK scaffold. Consistent with this model, the interaction between KSR2 and the core kinase components of the ERK cascade was confirmed by immunoblot analysis. As shown in Figure 1C, when KSR2 proteins were immunoprecipitated from NIH3T3 cell lines stably expressing KSR2, we found that MEK interacted constitutively with KSR2, whereas binding of B-Raf and ERK was strongly induced by growth factor treatment. Importantly, the MEK and ERK proteins associated with the KSR2 complex following growth factor treatment were in their activated state, indicating that binding to KSR2 supports the phospho-relay between Raf, MEK and ERK.

A key trait of KSR1 and other MAPK scaffolds is that their effect on cascade signaling is concentration dependent. Specifically, expression of the scaffold will enhance pathway activation when its levels are less than or equivalent to their binding partners, but will block pathway activation, when its levels exceed that of the interacting cascade components (Cacace et al., 1999; Kortum and Lewis, 2004; Levchenko et al., 2000). A sensitive assay for assessing the concentration effects of the KSR1 scaffold involves the Ras-dependent meiotic maturation of *Xenopus* oocytes. In this assay, oocyte maturation induced by activated Ras is dependent on ERK activation, and the kinetics of maturation will change when Ras to ERK signaling is modulated. As shown in Figure 1D, low expression of KSR2 (10 ng) accelerated the maturation of oocytes expressing Ras^{V12}, whereas high KSR2 expression (60 ng) blocked both oocyte maturation and ERK activation induced by Ras^{V12}, demonstrating a concentration-dependent effect. Thus, KSR2 interacts with the kinase components of the ERK cascade and facilitates ERK activation in a concentration-dependent manner, indicating that like KSR1, KSR2 functions as an ERK scaffold.

Calcineurin Selectively Interacts with KSR2

In addition to the known binding partners of KSR1, numerous other proteins involved in signal transduction were identified in the KSR complexes, including the guanine nucleotide exchange factor RapGEF6 and the protein kinases CK2, MARK2, and PKA (Figures 1B and S1, and Table S1). This approach was also able to distinguish proteins that appear to have a higher binding affinity for one KSR scaffold over the other. For example, the mass spectrometry

analysis revealed that the KSR2 complexes were notably enriched for proteins involved in the ubiquitination process and, in comparison to KSR1, contained an increased number of peptides derived from the E3 ligases Praja-2 and EDD1 as well as from components of the Cullin 4 E3 ligase complex including Cullin 4A, Cullin 4B DDB1, and VprBP/DCAF1 (Figures 1B and S1, and Table S1). Most significantly, the mass spectrometry analysis identified various proteins that interact selectively with KSR2 (Figure 1B). In particular, several of the KSR2-specific binding partners were proteins known to be involved in Ca^{2+} signaling, with the Ca^{2+}/calmodulin-regulated serine/threonine phosphatase calcineurin being the most abundant of the KSR2-specific interactors.

To confirm that calcineurin binds selectively to KSR2, KSR1 and KSR2 complexes were examined by immunoblot analysis for the presence of the catalytic (CN-A) and regulatory (CN-B) subunits of calcineurin. As a control, the complexes were also probed for the presence of the catalytic subunit of PP2A (PP2Ac), a protein detected in both KSR1 and KSR2 complexes. As shown in Figure 2A, although PP2Ac interacted with both KSR1 and KSR2 proteins containing the N-terminal region, the CN-A and CN-B subunits were only detected in complexes that contained the N-terminal region of KSR2. Analysis of KSR2 complexes isolated from mouse brain tissue confirmed the interaction of endogenous KSR2 and calcineurin under physiological conditions (Figure 2B upper panel). In addition, the calcineurin/KSR2 interaction was found to be constitutive, but could be disrupted by treating cells with the calcineurin inhibitor cyclosporin A (CsA; Figure 2B lower panel).

To further define the region of KSR2 required for calcineurin binding, a series of KSR2 truncation and deletion mutants were examined for their ability to associate with the CN-B subunit. As shown in Figure 2C, CN-B strongly interacted with the N′640, N′413 and C′301 truncation mutants, indicating that residues 301–413 were required for calcineurin binding. This region contains the 63 amino acid insert unique to KSR2 and deletion of this insert region (Δ329-401-KSR2) dramatically reduced calcineurin binding (Figure 2D). When the insert region was examined for the presence of a known calcineruin binding motif, such as the PxIxIT or LxVP motif (Dodge and Scott, 2003; Garcia-Cozar et al., 1998; Martinez-Martinez et al., 2006; Park et al., 2000), an LxVP motif was identified at amino acid positions 390–393 (LSVP). Subsequent mutation of the LxVP motif to alanine residues (LxVPm-KSR2) significantly disrupted the KSR2/calcineurin interaction, whereas mutation of the arginine residue adjacent to this motif (R394A-KSR2) had no effect (Figure 2D). In addition, deletion of the KSR2-specific insert or mutation of the LxVP motif did not alter the KSR2/PP2A interaction (Figure 2D), indicating that binding of the calcineurin and PP2A phosphatases to KSR2 occurs independently.

To confirm the specificity of the KSR2/calcineruin interaction, we next evaluated the ability of GST fusion proteins encoding the 303-413 region of KSR2 to bind calcineurin in protein pull-down assays. As shown in Figure 2E, CN-B strongly associated with the WT GST-303-413 protein, but failed to interact with a similar fusion protein containing alanine substitutions at the LxVP motif (GST-303-413 LxVPm; Figure 2E). Moreover, when the KSR2 sequences were cleaved from GST, concentrated and added to in vitro calcineurin assays, the WT 303-413 peptide reduced calcineurin activity in a dose dependent manner, whereas addition of the purified LxVPm peptide had little effect (Figure 2E). Similar results have been observed for peptides encoding the LxVP motif of NFATc (Martinez-Martinez et al., 2006), a well-documented substrate of calcineurin (Crabtree, 2001; Hogan et al., 2003), and it is thought that high affinity binding to these peptides reduces calcineurin's access to the labeled substrate. Together these findings identify KSR2 as a specific calcineurin binding partner and confirm that the interaction is mediated primarily by the LxVP motif on KSR2.

Ca^{2+}-Signaling Modulates KSR2 Localization and Scaffold Activity

To begin to address the functional significance of the KSR2/calcineurin interaction, we first compared the ERK scaffolding activity of KSR1, WT-KSR2, and LxVPm-KSR2 under conditions known to modulate Ca^{2+} levels and calcineurin activity. For these studies, the proteins were expressed in COS-7 cells, which lack endogenous KSR2, and the ERK scaffold activity was determined by assessing the level of active phosphoERK present in the KSR complexes and in cell lysates. To generate high levels of intracellular Ca^{2+} as well as mediate Ras pathway activation, cells were treated with the calcium ionophore ionomycin for 15 min and then stimulated with PMA for 5 min. WT-KSR2 complexes isolated from cells treated with PMA/ionomycin contained significantly higher levels of phosphoERK than did KSR1 or LxVPm-KSR2 complexes, and the level of activated ERK detected in cell lysates was found to correlate with the level of phosphoERK present in the various KSR complexes (Figures 3A top panel and S2A). Similar results were observed when cells were treated with histamine to elevate intracellular Ca^{2+} levels under more physiological conditions (Figure 3A middle panel). Treatment of cells with CsA to inhibit calcineurin activity prior to PMA/ionomycin or histamine stimulation greatly reduced the level of active ERK present in KSR2 complexes, but had little effect on those observed in either KSR1 or LxVPm-KSR2 complexes (Figures 3A top and middle panels and S2A). Notably, when cells were treated with EGF to induce ERK cascade signaling via RTK activation, high levels of phosphoERK were observed in all the KSR complexes and CsA treatment had no detectable effect on ERK activation (Figure 3A bottom panel).

The above findings suggest that calcineurin activity is not required for the scaffolding function of KSR2 induced by RTK activation, but may contribute to its function under conditions where signaling is strongly impacted by changes in Ca^{2+} levels. To further explore this possibility, we compared the subcellular localization of WT-KSR2 with that of LxVPm-KSR2 and KSR1 under the signaling conditions described above (Figure 3B). By immunofluorescent staining, all of the KSR proteins were found to localize in the cytosol of serum-starved cells, and as expected, translocated to the cell surface when RTK activation was induced by EGF treatment (Figure 3B). A pronounced relocalization of WT-KSR2 to the cell surface was also observed when cells were treated with either PMA/ionomycin or histamine, and a significant amount of active phosphoERK was found to colocalize with WT-KSR2 at the plasma membrane in cells stimulated with PMA/ionomycin (Figure 3B, C). In contrast, PMA/ionomycin or histamine treatment had only a weak effect on the localization of KSR1 or LxVPm-KSR2. Pretreatment of cells with CsA largely blocked the strong membrane localization of KSR2 induced by PMA/ionomycin or histamine stimulation, indicating that calcineurin activity contributes to the relocalization of KSR2 under these conditions.

KSR2 is a Substrate of Calcineurin

Previous studies have shown that phosphorylation-dependent 14-3-3 binding interactions play an important role in regulating the intracellular localization of the KSR1 scaffold. More specifically, KSR1 is retained in the cytosol of quiescent cells by the binding of a 14-3-3 dimer to two phosphoserine sites, pS297 and pS392. In response to RTK activation, PP2A has been found to dephosphorylate the pS392 binding site, resulting in the release of 14-3-3 from this site and the exposure of the C1 domain that is required for membrane targeting (Ory et al., 2003). Because the above results indicate that calcineurin activity is required for the membrane localization of KSR2 in response to Ca^{2+} signals, we next examined whether KSR2 might be a substrate of calcineurin. For these studies, the in vivo phosphosphorylation state of WT- and LxVPm-KSR2 was determined by metabolic [^{32}P]-labeling experiments and mass spectrometry analysis. As shown in Figure 4A, the HPLC profile of WT and LxVPm proteins labeled in serum-starved COS-7 cells indicated that both proteins contained six tryptic phosphopeptides, which through phosphoamino acid analysis, Edman degradation, mutational

analysis, and mass spectrometry were determined to represent peptides phosphorylated on S198 (fr. 11), T287 (fr. 26–27), S310 (fr. 34), S469 (fr. 42), S623 (fr. 99), and a peptide phosphorylated at two sites S485 and T492 (fr. 48). As predicted, the S310 and S469 phosphorylation sites were found to mediate 14-3-3 binding, and mutation of both residues was required to disrupt the 14-3-3/KSR2 interaction (Figure 4B).

When cells were treated with PMA/ionomycin, an 8–10% reduction in the phosphorylation status of the S469 14-3-3 binding site was observed for both WT- and LxVPm-KSR2 proteins and no change in the phosphorylation state of S485, T492 or S623 was observed for either protein (Figure 4A). Strikingly, the phosphorylation status of S198, T287 and the S310 14-3-3 binding site was reduced >60% for WT-KSR2, whereas minimal dephosphorylation of these sites was observed for LxVPm-KSR2 (Figure 4A). The dephosphorylation of S198 T287 and S310 induced by PMA/ionomycin treatment was largely blocked by pretreatment with CsA, whereas these sites were still dephosphorylated in cells pretreated with the PP2A inhibitor okadaic acid (Figures 4A and S4). It should be noted that S198 and T287 are sites followed by a proline residue and that a portion of these results could also be demonstrated using an antibody that recognizes phosphoS/TP sites (Figure S3A, B). Interestingly, we also found that the phosphorylation state of KSR2 was altered by RTK activation; however, in this case, EGF treatment primarily induced the dephosphorylation of the S469 14-3-3 binding site (Figure S4). The EGF-mediated dephosphorylation of S469 was blocked by okadaic acid treatment but not by CsA treatment, suggesting that this site is dephosphorylated by PP2A.

As a control for these studies, the phosphorylation state of KSR1was also examined. Consistent with previous studies (Ory et al., 2003), we found that KSR1 was highly phosphorylated on S297, S392, and S518 in quiescent cells and that RTK activation induced a significant reduction in the phosphorylation state of the pS392 14-3-3 binding site (Figures 4A and S4). In contrast, treatment of cells with PMA/ionomycin caused only minimal changes in KSR1 phosphosphorylation and the changes observed were not impacted by CsA treatment (Figure 4A). These findings indicate that calcineurin modulates the phosphorylation state of KSR2, but not KSR1, and identifies S198, T287 and the S310 14-3-3 binding site as the KSR2 residues targeted by calcineurin.

To address whether calcineurin-mediated dephosphorylation alters the subcellular localization of KSR2, the localization of KSR2 proteins in which the S198, T287 and/or S310 phosphorylation sites were mutated to alanine was examined by immunofluorescent staining. As shown in Figure 4C, mutation of S198 or T287 had no effect on KSR2 localization; however, constitutive membrane localization was observed when the S310 14-3-3 binding site was mutated alone or in combination with the S198 and T287 sites (S198A/T287A/S310A). Thus, dephosphorylation of the S310 14-3-3 binding site provides a mechanism for how calcineurin can modulate KSR2 localization and function in response to Ca^{2+} signals.

Endogenous KSR2 is Regulated by Ca^{2+}-dependent Signaling

To confirm that KSR2 is a Ca^{2+}-regulated ERK scaffold, we looked for a cell system where KSR2 is expressed and where Ca^{2+}-signaling is known to mediate ERK activation. The expression profile of murine KSR2 as indicated by EST databases appears to be quite restricted with expression indicated in brain, bone marrow, ovary, and pancreas. Consistent with this prediction, we find that KSR2 is expressed in mouse brain and pancreatic tissues, and that endogenous KSR2 can be detected in the pancreatic β-cell line INS1 and the neuroblastoma cell line NG108 (Figure 5A). Notably, glucose treatment of INS1 cells has been shown to activate ERK in a manner that is dependent on Ca^{2+} release and calcineurin activity (Arnette et al., 2003; Khoo and Cobb, 1997), and the depolarization of NG108 cells by KCl treatment has been found to activate ERK in a Ca^{2+}-dependent manner (Schmitt et al., 2004). Therefore,

we used these two cell systems to further investigate the effect of Ca^{2+} signaling on KSR2 function.

As shown in Figure 5B, active phosphoERK was detected in endogenous KSR2 complexes isolated from either glucose-treated INS1 cells or KCl-treated NG108 cells, and the level of active phosphoERK present in these complexes was significantly reduced when cells were pretreated with CsA prior to stimulation. Similar results were also observed when Pyo-tagged KSR2 was exogenously expressed in INS1 cells (Figure S2B). By immunofluorescent staining, we found that both glucose treatment of INS1 cells and KCl-treatment of NG108 cells caused endogenous KSR2 to translocate to the cell surface in a manner that was dependent on calcineurin activity (Figure 5C). Metabolic labeling experiments further revealed that the phosphorylation state of endogenous KSR2 in quiescent INS1 and NG108 cells was similar to that observed for Pyo-tagged KSR2 isolated from quiescent COS-7 cells (Figure 5D). Glucose treatment of INS1 cells reduced the level of S198, T287 and S310 phoshphorylation ~60% and KCl treatment of NG108 cells caused a 90–100% reduction in the phosphorylation state of these sites. In both cell lines, the dephosphorylation of these sites could be largely blocked by CsA pretreatment. Together, these findings indicate that endogenous KSR2 is regulated by Ca^{2+}-dependent signaling and that KSR2 is a calcineurin target under physiological conditions.

Depletion of KSR2 Impairs Ca^{2+}-mediated ERK Activation and Signaling in INS1 and NG108 Cells

To investigate the importance of KSR2 to the Ca^{2+}-regulated signaling pathways described above, we initiated experiments examining the effect of KSR2 depletion. Using either transfected small interfering RNAs (siRNAs) or a lentivirus vector-based short-hairpin RNA (shRNA) directed against murine KSR2, we were able to achieve ≥90% knock-down of KSR2 protein levels in both INS1 and NG108 cells. As shown in Figure 6A, when NG108 cells were examined, we found that in comparison to control cells, KCl-mediated ERK activation was significantly reduced in cells depleted of KSR2. Interestingly, in INS1 cells, basal phosphoERK levels were elevated in KSR2-depleted cells; however, little increase in ERK activation was observed when cells were treated with glucose (Figure 6B). KSR2-depleted INS1 cells also had a slightly elevated basal level of insulin secretion in comparison to control cells, but exhibited an impaired response to glucose treatment (Figure 6B). These findings do not appear to be due to off-target effects of RNA interference, given that similar results were observed using either KSR2 siRNAs or shRNA. Moreover, the KSR1 scaffold is unlikely to be a major contributor to these effects, given that depletion of KSR1 was found to have little impact in either system (Figure 6A and B).

To further address the functional significance of the KSR2/calcineurin interaction, we next generated WT- and LxVPm-KSR2 proteins that were resistant to knock-down by the KSR2-shRNA vector. The resistant KSR2 proteins were then expressed in NG108 cells depleted of KSR2 by shRNA knock-down, following which the cells were evaluated for ERK activation and neurite formation. As shown in Figure 7A, re-expression of WT-KSR2 dramatically increased ERK activation induced by KCl treatment, whereas expression of the LxVPm mutant had a much more modest effect. Previous studies in NG108 cells have shown that ERK activation is required for KCl-mediated neurite outgrowth and that neurite formation can be inhibited by agents that reduce ERK signaling, such as U0126 treatment. Consistent with these reports, we found that in comparison to control cells, KSR2-depleted cells were not only impaired in KCl-mediated ERK activation, they were also impaired in their ability to form neurites in response to growth in 60 mM KCl (Figure 7B). Re-expression of WT-KSR2 in KSR2-depleted cells induced ~73% of cells to form neurites that were greater than one cell body in length, whereas ~28% of cells expressing LxVPm-KSR2 exhibited neurite outgrowth and the neurites that formed were much shorter than those observed in WT-KSR2-expressing

cells. These findings suggest that calcineurin binding is required for full KSR2 function under Ca^{2+} signaling conditions.

DISCUSSION

To gain a better understanding of how the mammalian KSR scaffolds contribute to ERK cascade signaling, we have take a proteomics approach to characterize the murine KSR1 and KSR2 scaffold complexes. This approach has revealed common functions and regulatory mechanisms for the KSR scaffolds and has allowed us to discern differences between the two scaffolds that might otherwise have gone undetected. Aided by this analysis, here we define a specific function for KSR2 in Ca^{2+}-mediated ERK cascade activation and signaling.

Analysis of the KSR scaffold complexes by mass spectrometry revealed that both complexes contained the core kinase components of the ERK cascade – Raf, MEK, and ERK, as well as many of the previously known regulators of KSR1. Subsequent biochemical analysis confirmed that like KSR1, KSR2 functions in a concentration-dependent manner to facilitate ERK cascade activation in vivo. Moreover, as has been observed for KSR1, KSR2 was found to translocate to the plasma membrane in response to growth factor-mediated RTK activation, indicating that both the murine KSR proteins can function as RTK-regulated ERK scaffolds.

In addition to the known binding partners, both KSR complexes contained other protein interactors that had not been previously described, including various molecules involved in signal transduction and the ubiquitination process. Significantly, despite the overall sequence homology and conserved domain structure of the KSR scaffolds, the proteomic approach identified several proteins that interact selectively with KSR2. Of the KSR2-specific interactors, calcineurin was the most abundant in KSR2 complexes. Binding of calcineurin was direct, mediated primarily by an LxVP motif on KSR2 similar to that found on the well-characterized substrates of calcineurin, the NFAT transcription factors. The LxVP motif is located at the end of the KSR2 unique insert region and is not present in mammalian KSR1 proteins, explaining why calcineurin is not found in KSR1 complexes.

Previous studies have shown that in response to Ca^{2+} signals, activated calcineurin dephosphorylates cytosolic NFAT proteins on numerous sites, allowing NFAT to translocate to the nucleus and function as a transcription factor. Likewise, our findings indicate that KSR2 is a substrate of calcineurin. From metabolic labeling experiments, we find that KSR2 is phosphorylated on at least seven sites in quiescent cells: S198, T287, S485, T492, S623 and the two 14-3-3 binding residues S310 and S469. Under conditions where Ca^{2+} levels are elevated, a significant dephosphorylation of S198, T287 and the S310 14-3-3 binding site was observed. Dephosphorylation of these sites was notably reduced for the LxVPm-KSR2 mutant that is defective in calcineurin binding and was blocked when CsA was used to inhibit calcineurin activity, indicating that both the interaction between KSR2 and calcineurin as well as calcineurin activity are required for the dephosphorylation events. In addition, we found that increased Ca^{2+} levels had no effect on the phosphorylation state of KSR1, which does not interact with calcineurin, demonstrating that calcineurin selectively regulates KSR2 phosphorylation.

As with the NFAT transcription factors, our findings indicate that calcineurin functions as a positive regulator of KSR2, allowing KSR2 to translocate to the plasma membrane and facilitate ERK activation in response to Ca^{2+} signals. In particular, the calcineurin-mediated dephosphorylation of the S310 14-3-3 binding site appears to be critical for the translocation of KSR2 to the cell surface, given that mutation of this residue results in constitutive membrane localization. Interestingly, RTK signaling was found to have little effect on the phosphorylation state of S198, T287 and the S310 14-3-3 binding site, but did induce the dephosphorylation of

the S469 14-3-3 binding site (a site analogous to the S392 14-3-3 binding site of KSR1 that has previously been shown to be dephosphorylated by PP2A in response to RTK activation). Treatment of cells with the PP2A inhibitor okadaic acid blocked the RTK-induced dephosphorylation of S469, but had no effect on the Ca^{2+}-induced dephosphorylation of S310. In contrast, inhibition of calcineurin by CsA treatment had the opposite effect. Taken together these findings suggest that RTK signaling engages PP2A to target the S469 14-3-3 binding site, whereas Ca^{2+} signaling activates calcineurin to target the S310 14-3-3 binding site and mediate the membrane recruitment of KSR2. Thus, the binding of PP2A to KSR1 and KSR2 may permit both proteins to function as RTK-regulated ERK scaffolds, whereas the selective binding of calcineurin to KSR2 might allow KSR2 alone to be responsive to Ca^{2+} signals (Figure 7C). Of note, the sole Drosophila KSR protein does contain an LxVP motif, suggesting that D-KSR may also be responsive to both RTK and Ca^{2+} signals.

In tissues where KSR2 is expressed, namely the brain and pancreas, it is known that Ca^{2+} can activate ERK cascade signaling (Dolmetsch et al., 2001; Rosen et al., 1994; Schmitt et al., 2004; Wu et al., 2001). Here, we find that endogenous KSR2 expression can be detected in INS1 pancreatic β-cells and in NG108 neuroblastoma cells and that in both cell lines, KSR2 function is modulated by Ca^{2+} signals. Specifically, glucose treatment of INS1 cells or depolarization of NG108 cells with KCl treatment caused the translocation of endogeneous KSR2 to the cell surface and promoted the association of active phosphoERK with KSR2. Moreover, these changes correlated with a dephosphorylation of endogenous KSR2 on the calcineurin-targeted sites. Further evidence that KSR2 is a relevant effector of Ca^{2+}-signaling in these systems, depletion of KSR2 significantly impaired ERK activation and insulin secretion in glucose-treated INS1 cells and reduced ERK activation and neurite outgrowth in KCl-treated NG108 cells. In NG108 cells, re-expression of WT-KSR2 was able to restore the defects associated with KSR2 depletion; whereas expression of the LxVPm mutant had only a modest effect, indicating the importance of calcineurin binding for full KSR2 activity.

Ca^{2+} functions as a critical signaling mediator in many biological processes, and although it has been well documented that Ca^{2+} can modulate Ras to ERK pathway activation, the mechanisms for how Ca^{2+} exerts this effect have not been fully elucidated. A number of Ca^{2+} responsive RasGEFs and RasGAPs have been identified (Cullen and Lockyer, 2002); however, a role for Ca^{2+} downstream of Ras has been unclear. Our findings identify KSR2 as a Ca^{2+}-regulated ERK scaffold and define another distinct mechanism whereby Ca^{2+} can impact Ras to ERK signaling. In light of recent findings that scaffolds play a important role in localizing active ERK to specific membrane microdomains and, as a result, to specific substrates (Casar et al., 2008), it will be of interest to determine whether Ca^{2+} signals recruit the KSR2 scaffold and active ERK to the same membrane microdomain as does RTK signaling. In addition, whether the recruitment of KSR2 to such a membrane microdomain might localize calcineurin to other critical substrates involved in Ca^{2+} signaling is also of interest and awaits further investigation.

EXPERIMENTAL PROCEDURES

Antibodies and Reagents

A KSR2-specific rabbit antibody was generated against residues 2–15 of murine KSR2 and a KSR1-specific rabbit antibody has been previously described (Cacace et al., 1999). A KSR2-specific monoclonal antibody generated against residues 411–500 of human KSR2 was also purchased from Novus. Antibodies recognizing phosphoERK, phosphoMEK and phosphoS/TP were from Cell Signaling Technologies, antibodies to MEK and PP2Ac were from BD Biosciences, antibodies to 14-3-3, B-Raf, and CN-A were from Santa Cruz, PDGF and CN-B antibody were obtained from Millipore, and EGF was from Invitrogen. Calcineurin, calmodulin, histamine, and thrombin were purchased from Sigma. Ionomycin, PMA,

cyclosporin A, and thapsigargin were from Calbiochem/EMD Biosciences. Smartpool siRNA duplexes designed against rat KSR1 or mouse KSR2 were purchased from Dharmacon and the pLKO.1 murine KSR2 shRNA vector was obtained from Open Biosource.

Mass Spectrometry Analysis of KSR1 and KSR2 Protein Complexes

Cycling 293T cells expressing Pyo-KSR1 or Pyo-KSR2 were lysed in Triton X-100 lysis buffer (20mM Tris [pH 8.0], 50 mM NaCl, 10% glycerol, 1% Triton X-100, 0.15 U/ml aprotinin, 1 mM PMSF, 20uM leupeptin, and 5 mM sodium vanadate) and the Pyo-tagged KSR proteins were isolated and analyzed by mass spectrometry as previously described (Ory et al., 2003).

Metabolic Labeling and Phosphorylation Site Mapping

COS-7 cells expressing Pyo-KSR2 constructs, INS1 or NG108 cells were incubated for 4–6 hr at 37 °C in phosphate-free DMEM containing 2.5% dialyzed calf serum and [^{32}P] orthophosphate (1 mCi/ml media). Cells were stimulated with the appropriate agent prior to lysis. Labeled KSR2 proteins were immunoprecipitated from cell lysates, separated by SDS-PAGE, eluted from the gel matrix, digested with trypsin, and analyzed by reverse-phase HPLC, phosphoamino acid analysis, and Edman degradation (Morrison et al., 1993).

Depletion of KSR Proteins

For siRNA-mediated depletion, Dharmacon Smartpool siRNA oligonucleotides designed against rat KSR1 or mouse KSR2 were transfected into INS1 and NG108 cells using DharmaFECT1. Cells were then evaluated 72 hr after transfection. For shRNA-mediated depletion, a lentivirus-based pLKO.1 plasmid expressing murine KSR2 shRNA was used. Viral particles were generated with the MISSION lentiviral packaging mix (Sigma) and used to establish heterogeneous populations of INS1 or NG108 cells stably expressing the KSR2 shRNA.

Glucose-stimulated Insulin Secretion Assay

WT or KSR2 depleted INS1 cells were grown to confluency in 60 mm dishes. 18 hours prior to the assay, the culture media was replaced with fresh RPM1 1640 containing 2.5 mM glucose and no serum. On the day of the assay, cells were washed twice with HEPES balanced salt solution (HBSS, 114 mM NaCl, 470 mM KCl, 1.2 mM KH_2PO_4, 1.16 mM $MgSO_4$, 20 mM HEPES, 2.5 mM $CaCl_2$, 25.5 mM $NaHCO_3$, 0.2% BSA, pH 7.0) and quiesced for 2 hr in HBSS containing 2.5 mM glucose. The HBSS buffer was then removed and 2mL fresh HBSS containing either 2.5 mM or 15 mM glucose was added to the cells. After incubation for 2hr, the buffer was removed from the cells and insulin levels determined using the Coat-A-Count Insulin RIA kit (Siemens).

Neurite Differentiation

Control or KSR2-depleted NG108 cells were seeded into 60 mM dishes (3.0×10^5 cells/dish) containing acid washed glass coverslips in DMEM supplemented with 1% fetal bovine serum. 18 hr after plating, plasmids expressing either GFP or the KSR2-shRNA resistant Pyo-tagged WT or LxVPm-KSR2 proteins were transfected into cells. Following transfection, cells were treated with 60mM KCl for 3–4 days prior to examination by immunofluorescent staining.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank members of the Laboratory of Cell and Developmental Signaling for helpful discussions and comments. This project was supported with federal funds from the National Cancer Institute, National Institutes of Health, DHHS, in part under Contract N01-CO-12400.

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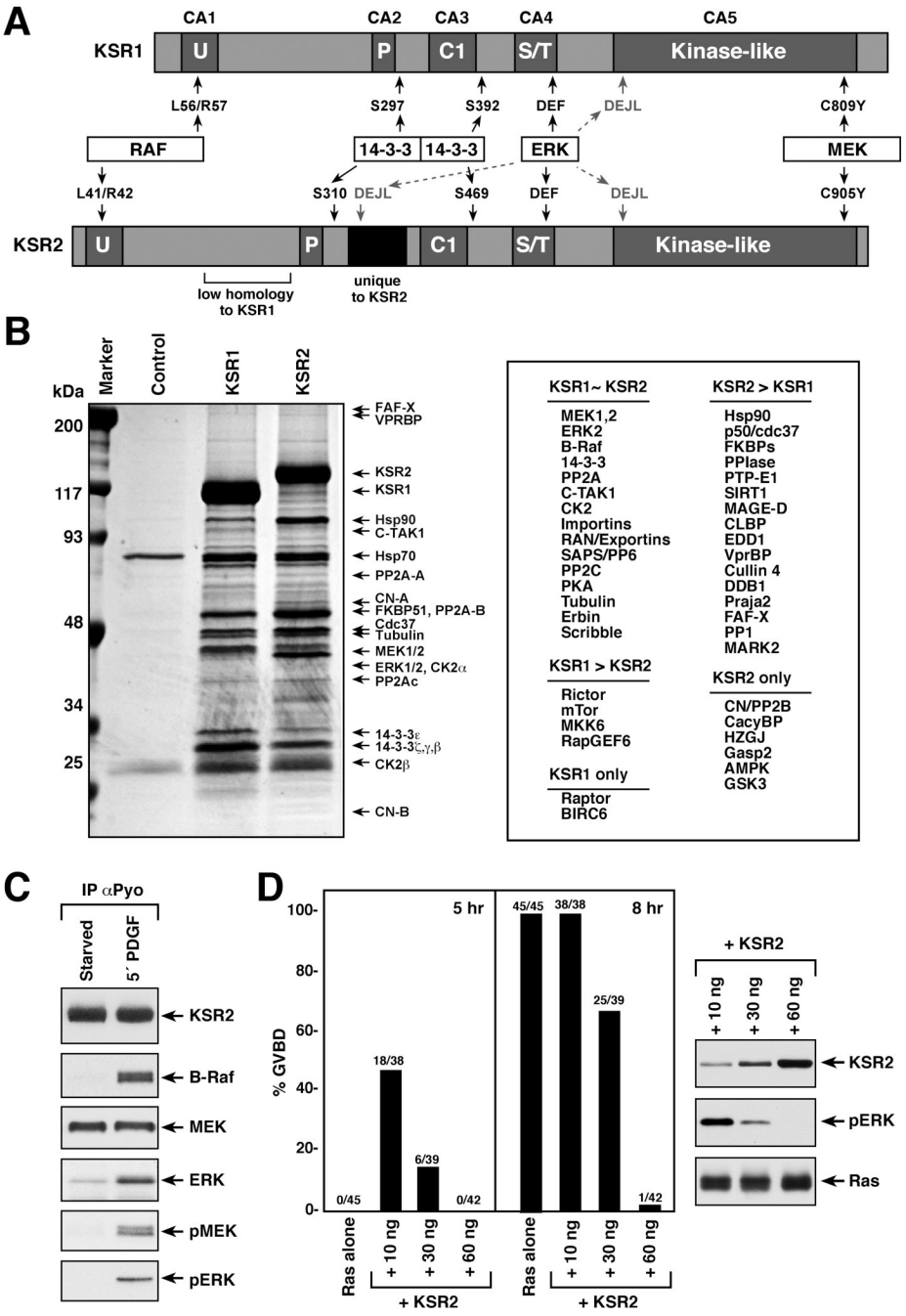


Figure 1.

KSR2 Functions as a MAPK Scaffold for the ERK Cascade

(A) Schematic depiction of KSR1 and KSR2. Amino acids and motifs involved in binding interactions are indicated as are the five conserved KSR domains (CA1-5). (B) KSR complexes isolated from cycling 293T cells were resolved by SDS-PAGE and stained with Coomassie brilliant blue. The control samples were prepared from untransfected cell lysates. Various proteins identified in the KSR complexes are indicated. (C) Pyo-KSR2 complexes were isolated from serum-starved or PDGF-treated NIH3T3 cells and examined for the presence of B-Raf, MEK, ERK, active pMEK and active pERK by immunoblot analysis. (D) Stage VI *Xenopus* oocytes were injected with the indicated amount of mRNA encoding KSR2. Oocytes

were later injected with 3 ng of Ras^{V12} mRNA and scored for maturation (as determined by germinal vesicle breakdown; GVBD) 5 and 8 hr following Ras injection. Oocyte lysates were prepared at the 8 hr time point and examined by immunoblot analysis as indicated.

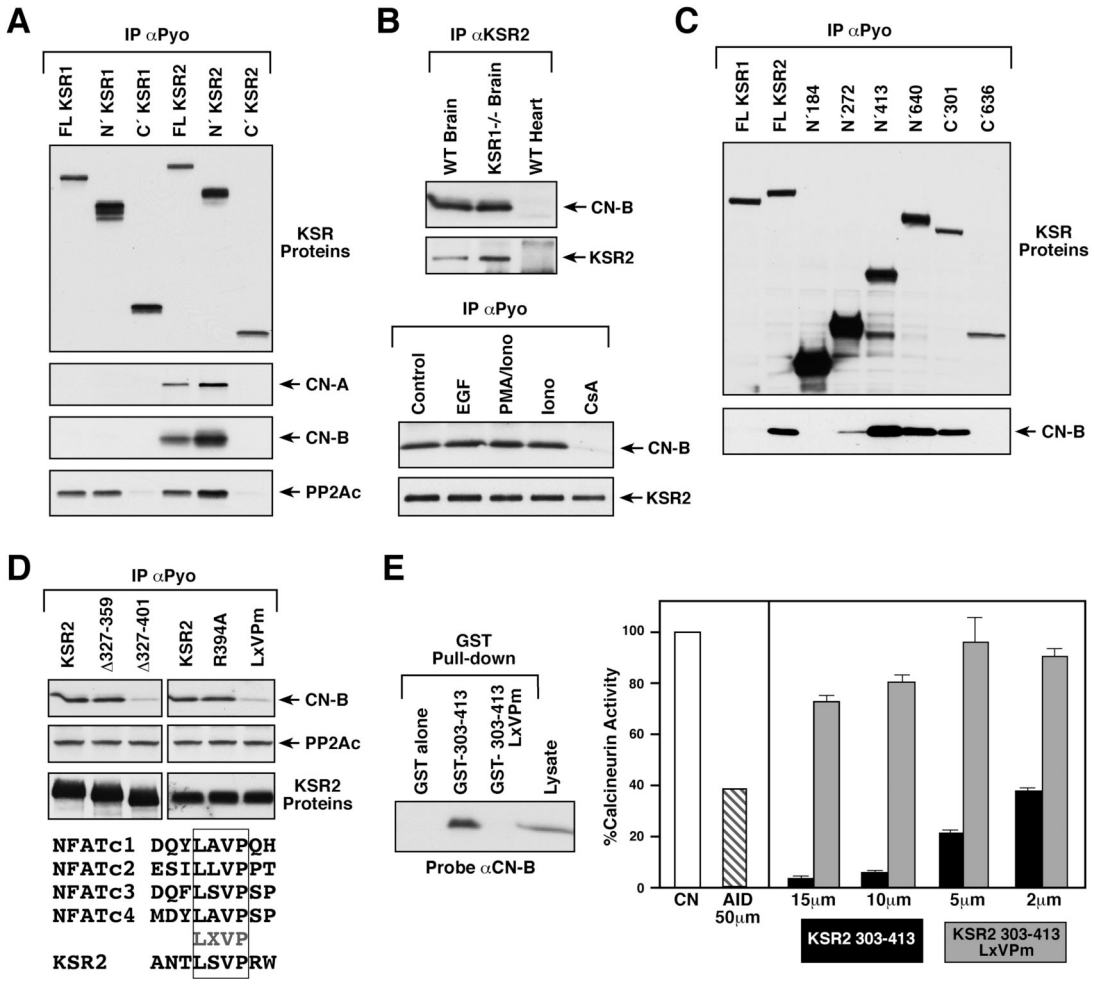


Figure 2.

Calcineurin Selectively Interacts with KSR2

(A) Pyo-tagged full-length (FL) and truncated N- and C-terminal KSR1 and KSR2 proteins were isolated from cycling COS-7 cells and examined for the presence of PP2Ac, CN-A and CN-B.

(B) Endogenous KSR2 complexes isolated from brain tissue of WT or KSR1$^{-/-}$ mice were examined for the presence of CN-B (upper). Serum starved COS-7 cells expressing Pyo-KSR2 were treated with 100 ng/ml EGF (5 min), 1 μM ionomycin (15 min)/50 ng/ml PMA (5 min), 1 μM ionomycin (15 min) or 5 μM CsA (15 min). KSR2 complexes were isolated and examined for the presence of CN-B (lower). (C and D) Various Pyo-KSR2 mutant complexes were isolated from cycling COS-7 cells and examined for the presence of CN-B or PP2Ac. Sequence alignment of the LxVP motif found in NFAT proteins and KSR2. (E) GST-tagged KSR2 303-413 or KSR2 303-413-LxVPm proteins were examined for their ability to bind CN-B. Calcineurin was incubated with the indicated peptides and activity assays were performed using phosphorylated RII as a substrate. KSR2 peptides were cleaved from GST and AID is a peptide derived from the autoinhibitory domain of calcineurin. Results represent the range of two independent experiments performed in triplicate.

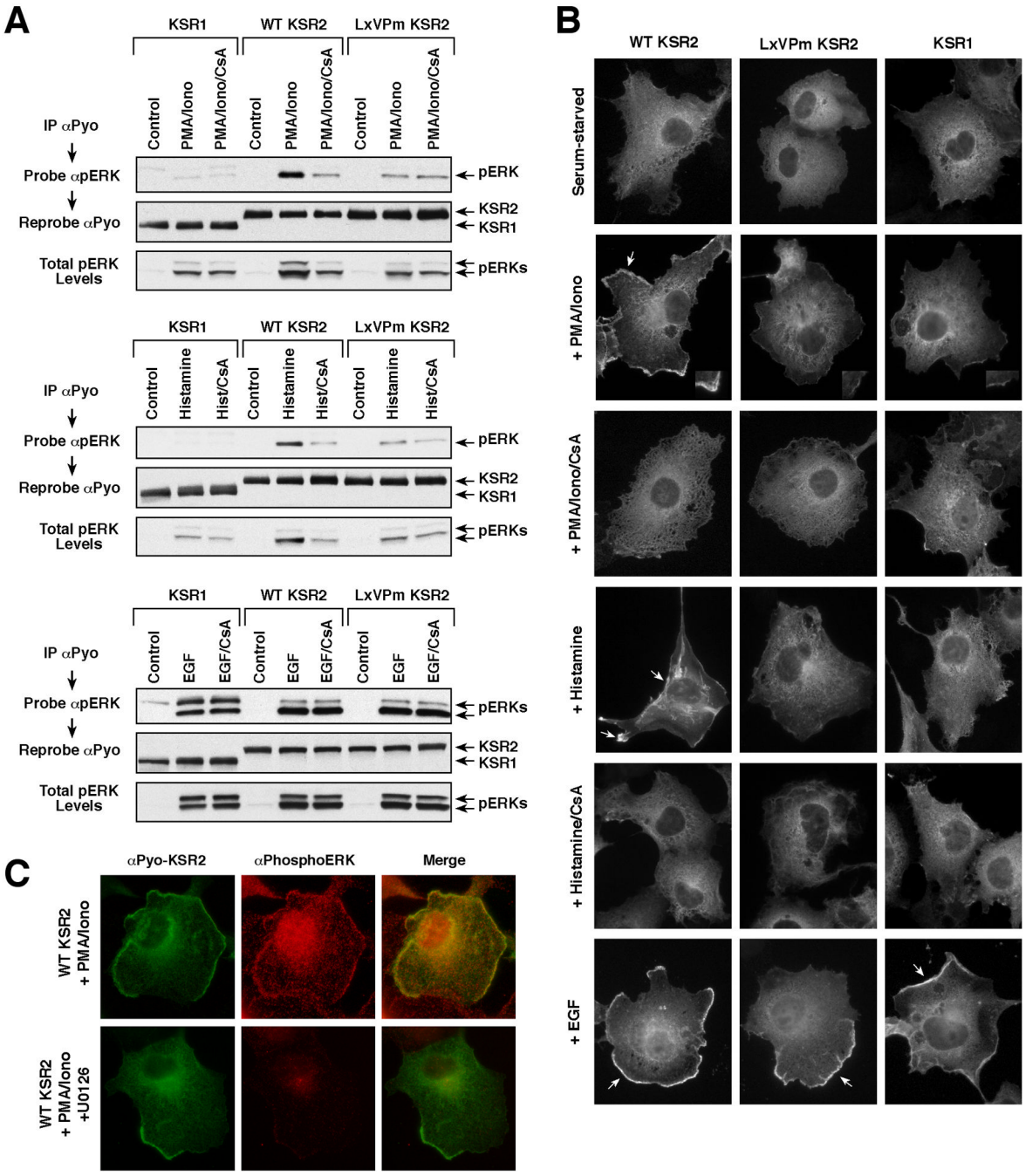


Figure 3.

Ca^{2+}-signaling Modulates the Scaffolding Activity and Localization of KSR2

(A) Serum-starved COS-7 cells expressing Pyo-tagged WT-KSR2, LxVPm-KSR2 or KSR1 were treated as indicated. KSR complexes and total cell lysates were examined for the presence of active pERK. (B and C) Serum starved COS-7 cells expressing WT-KSR2, LxVPm-KSR2, or KSR1 were treated as indicated prior to fixation. Localization of the KSR proteins and active pERK was determined by immunofluorescent staining.

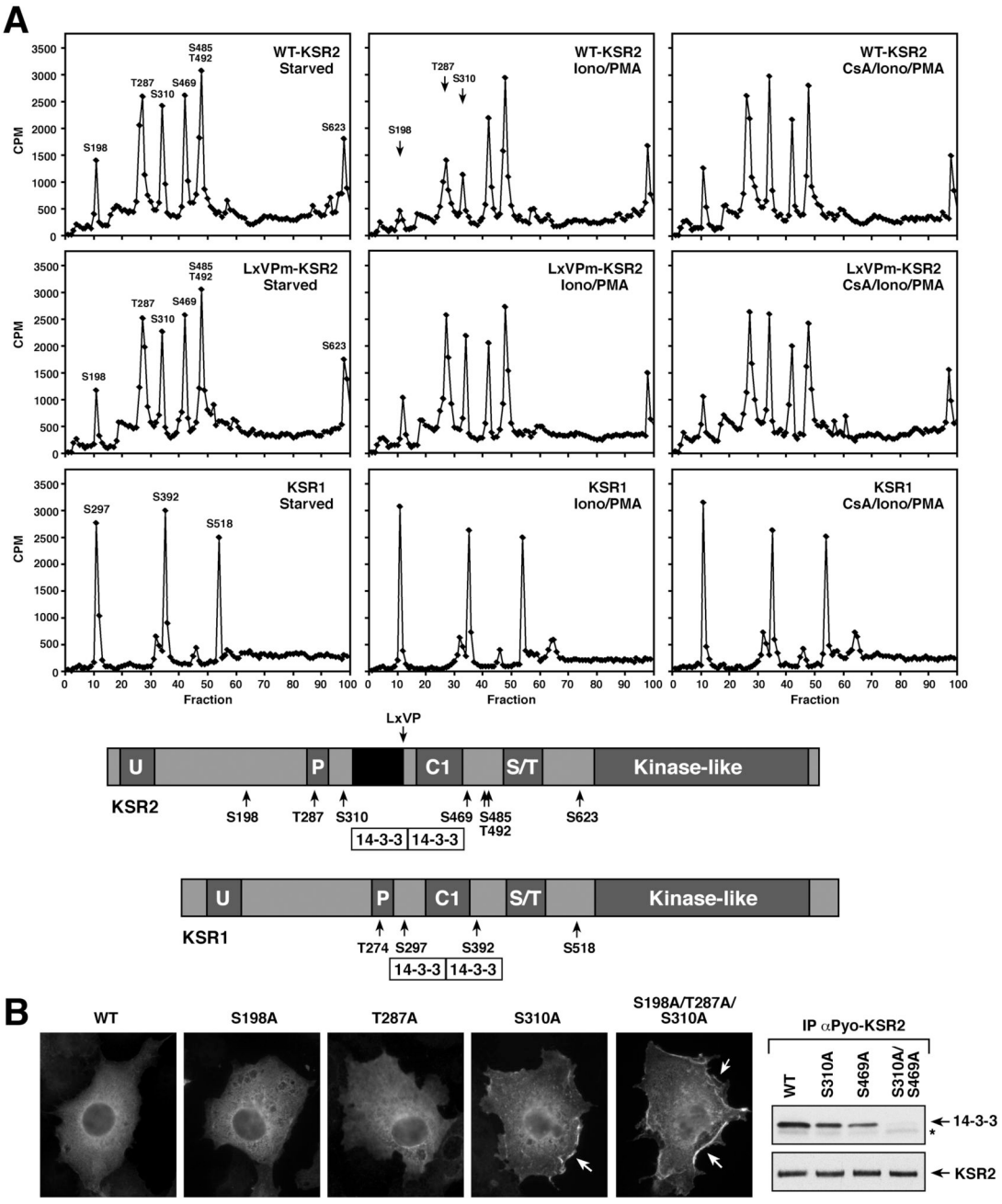


Figure 4.

KSR2 is a Calcineurin Substrate

(A) Quiescent COS-7 expressing Pyo-tagged WT-KSR2, LxVPm-KSR2, or KSR1 were metabolically labeled with [^{32}P]orthophosphate. Untreated or CsA-treated cells were stimulated with PMA/ionomycin prior to lysis. Immunoprecipitated KSR proteins were digested with trypsin and the tryptic phosphopeptides were separated by HPLC. Shown are the profiles of radioactivity collected in the HPLC fractions. Phosphorylated residues are indicated. (B) Localization of Pyo-KSR2 proteins in cycling COS-7 cells was determined by immunofluorescent staining. Pyo-KSR2 mutant complexes were isolated from cycling COS-7 cells and examined for the presence of 14-3-3.

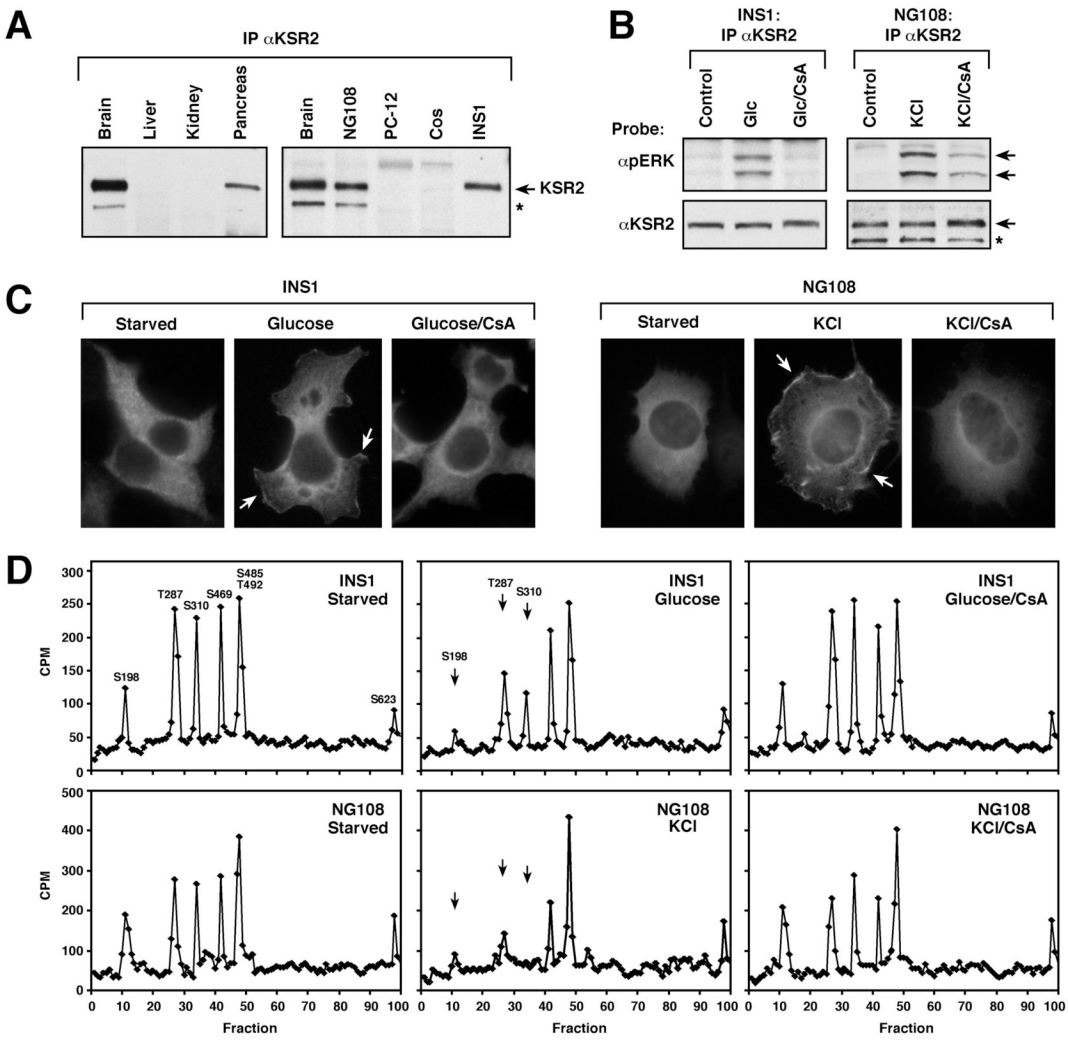


Figure 5.

Endogenous KSR2 is Regulated by Ca^{2+} Signals in INS1 and NG108 Cells

(A) Lysates prepared from various mouse tissues and cell lines were examined for endogenous KSR2 expression. * indicates non-specific band. (B) Untreated or CsA-treated NG108 and INS1 cells were stimulated with either glucose (Glc) or KCl for 20 min. Endogenous KSR2 complexes were isolated and examined for the presence of active pERK. (C) Localization of endogenous KSR2 in INS1 and NG108 cells was determined by immunofluorescent staining. (D) INS1 and NG108 cells were metabolically labeled with [^{32}P]orthophosphate and treated as indicated. Endogenous KSR2 proteins were isolated and examined by HPLC analysis.

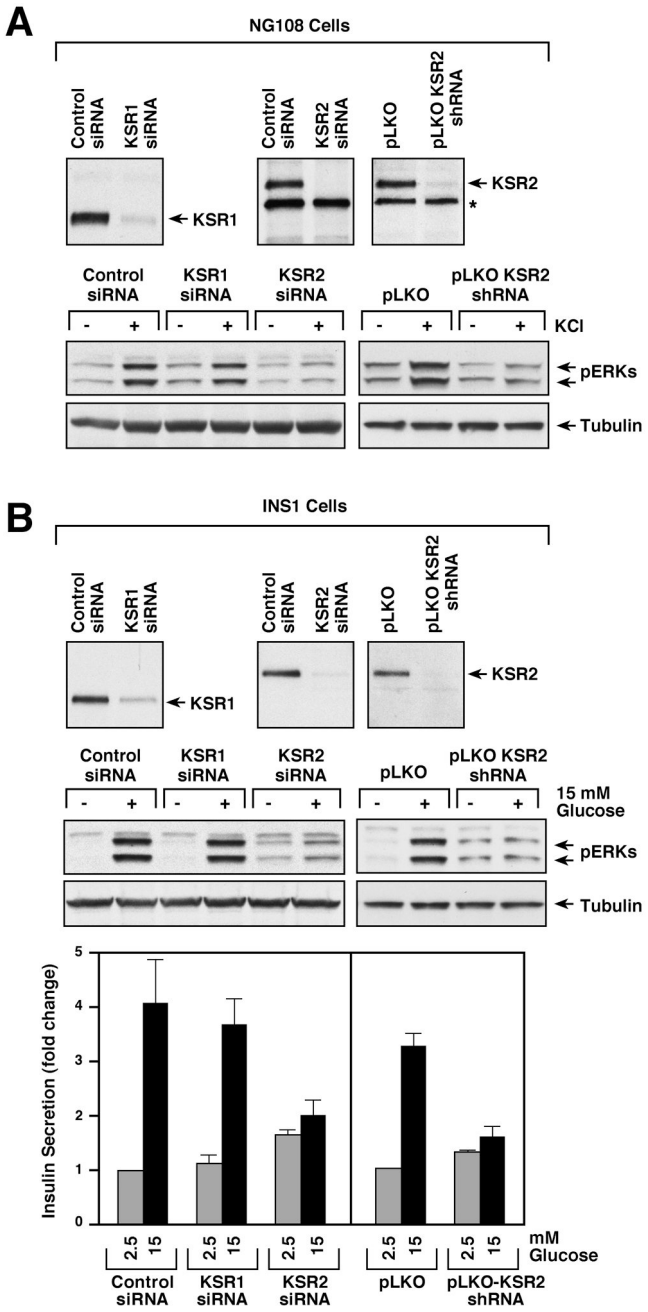
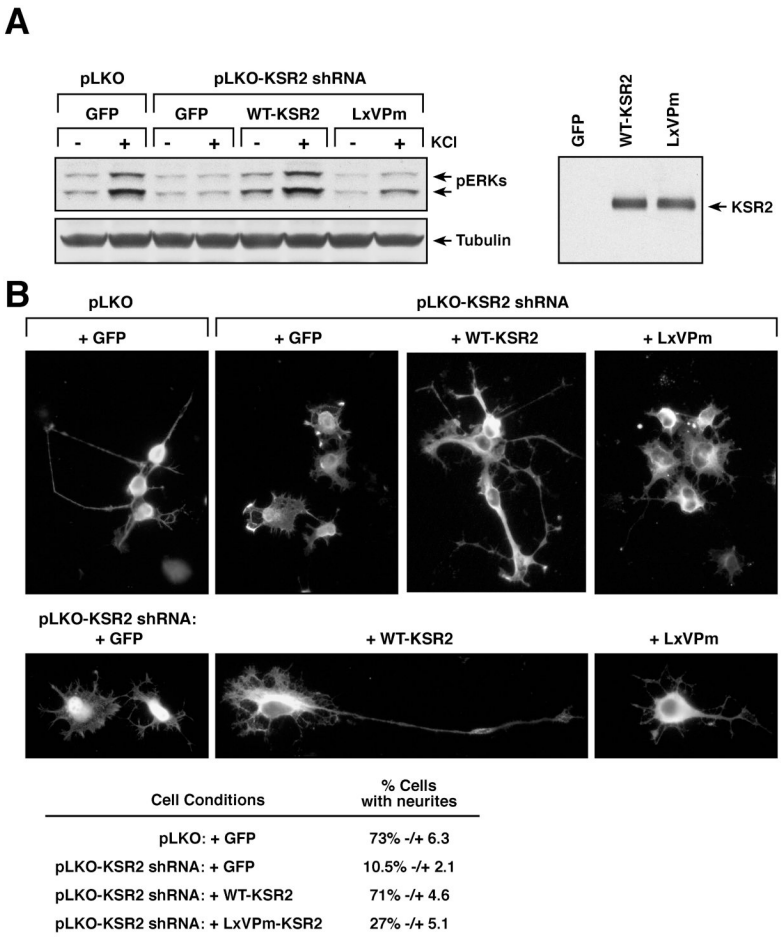


Figure 6.

Depletion of KSR2 Impairs Ca^{2+}-mediated ERK Signaling in NG108 and INS1 Cells (A) NG108 cells transfected with control, KSR1- or KSR2-siRNAs were treated as indicated and then examined for KSR and pERK levels. Lentivirus-infected cells expressing either the pLKO vector or pLKO-KSR2 shRNA were also examined. Tubulin levels are shown as a protein loading control. (B) Control and KSR1- or KSR2-depleted INS1 cells were examined as in (A). Cells were also were examined for insulin secretion following culture for 2 hr in media containing either 2.5 or 15 mM glucose. Results represent the range of three independent experiments performed in duplicate.



Cell Conditions	% Cells with neurites
pLKO: + GFP	73% -/+ 6.3
pLKO-KSR2 shRNA: + GFP	10.5% -/+ 2.1
pLKO-KSR2 shRNA: + WT-KSR2	71% -/+ 4.6
pLKO-KSR2 shRNA: + LxVPm-KSR2	27% -/+ 5.1

Figure 7.

Calcineurin Binding is Required for Full KSR2 Function in KCl-treated NG108 Cells NG108 cells expressing pLKO-KSR2 shRNA were transfected with plasmids encoding either GFP or resistant Pyo-tagged WT- or LxVPm KSR2. Cells were then examined for pERK and KSR2 levels following treatment with 60 mM KCl for 20 min (A) or for neurite formation following growth for 3 days in media containing 60 mM KCl (B). As a control, NG108 cells expressing the pLKO vector were also examined.