The Functional Interaction of 14-3-3 Proteins with the ERK1/2 Scaffold KSR1 Occurs in an Isoform-specific Manner*

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Identifying 14-3-3 isoform-specific substrates and functions may be of broad relevance to cell signaling research because of the key role played by this family of proteins in many vital processes. A multitude of ligands have been identified, but the extent to which they are isoform-specific is a matter of debate. Herein we demonstrate, both in vitro and in vivo, a specific, functionally relevant interaction of human 14-3-3 with the molecular scaffold KSR1, which is mediated by the C-terminal stretch of 14-3-3γ. Specific binding to 14-3-3γ protected KSR1 from epidermal growth factor-induced dephosphorylation and impaired its ability to activate ERK2 and facilitate Ras signaling in Xenopus oocytes. Furthermore, RNA interference-mediated inhibition of 14-3-3γ resulted in the accumulation of KSR1 in the plasma membrane, all in accordance with 14-3-3γ being the cytosolic anchor that keeps KSR1 inactive. We also provide evidence that KSR1-bound 14-3-3γ heterodimerized preferentially with selected isoforms and that KSR1 bound monomeric 14-3-3γ. In sum, we have demonstrated ligand discrimination among 14-3-3 isoforms and shed light on molecular mechanisms of 14-3-3 functional specificity and KSR1 regulation.

The 14-3-3 proteins comprise a large family of highly conserved, acidic polypeptides of 28–33 kDa that are expressed ubiquitously in all eukaryotic species (1). Seven isoforms, each encoded by a distinct gene, have been described in mammals: β, γ, δ, ε, η, σ, θ/β, and ζ. In plants, up to 15 isoforms have been identified, and in yeast, Drosophila melanogaster and Caenorhabditis elegans, only two isoforms have been reported (1). Initially described in 1967 (2), these proteins were characterized a decade ago as the first distinct phosphoserine (pSer)-binding proteins (3). Since then, a varied multitude of interacting partners have been identified, participating in cellular processes as diverse as signal transduction, cell-cycle control, apoptosis, regulation of metabolism, protein trafficking, cell morphology, transcription, stress response, and oncogenic transformation (1, 4), thereby highlighting 14-3-3 proteins as key mediators of intracellular signaling. Large scale analyses aimed at identifying potential 14-3-3 ligands have consistently resulted in long lists of proteins. Two laboratories independently have identified more than 200 interacting partners using in vitro affinity chromatography protocol (5, 6), and a recent direct proteomic analysis has identified as many as 170 specific 14-3-3-interacting proteins (7). Further, transgenic mouse proteomics allowed the identification of 147 brain proteins interacting with 14-3-3ζ (8). With the seven mammalian isoforms sharing a 70% identity, the question arises as to how they achieve specificity in regulating hundreds of different proteins.

On a structural level, 14-3-3 proteins form U-shaped dimers, each monomer containing nine anti-parallel α-helices, named A to I (9). Helices A to D are involved in dimer formation, and helices C, E, G, and I form a large amphipathic groove critically involved in binding to pSer/Thr-containing proteins (9). Screening of phosphopeptide libraries and structural analysis of 14-3-3 phosphopeptide complexes have identified two high-affinity binding motifs: RXpSXP (mode 1) and RXXXpSXP (mode 2) (10). In addition, exoenzyme S is able to interact with 14-3-3 in a nonphosphorylated form (11), and recently a C-terminal mode 3-binding motif has been described in some 14-3-3 ligands having a general consensus of pS/T)X2−1−2-COOH (12). Despite these exceptions, the majority of ligands bind to 14-3-3 proteins through the unique mode 1 or mode 2 sequence. This, in addition to extensive sequence conservation among 14-3-3 proteins, makes it difficult to uncover any specific role for each isoform and to understand the molecular determinants con-
We show that the flexible C-terminal tail of 14-3-3 specifically with KSR1, regulating its ability to translocate to the cytosol until Ras activation. It is not known whether KSR1 interacts promiscuously with 14-3-3 proteins or, on the contrary, the interaction is isoform-specific. Therefore, we chose KSR1 as a functionally relevant ligand to study 14-3-3 isoform specificity and to gain insight into its own regulation.

Here, we demonstrate the existence of functional specificity among 14-3-3 isoforms by showing that 14-3-3-3 binds specifically with KSR1, regulating its ability to translocate to the plasma membrane and facilitate Ras-induced ERK2 activation. KSR1 also interacts with regulatory enzymes such as the Ser/Thr phosphatase PP2A (19) and the Ser/Thr kinases C-TAK1 (Cdc25C-associated kinase) and CK2 (casein kinase 2) (20, 21). Binding of KSR1 to 14-3-3 is regulated by phosphorylation on Ser-392 by C-TAK1 and on Ser-297 by an unknown kinase (17). The KSR1-bound PP2A activity dephosphorylates pSer-392 in response to Ras activation, resulting in the dissociation of the KSR1/14-3-3 complex and translocation of KSR1 to the plasma membrane, likely mediated by exposure of its cysteine-rich domain (CRD) (19). In the membrane, KSR1 facilitates ERK activation by a dual mechanism involving the assembling of a signaling complex (scaffold function) and activation of Ras kinases through its associated CK2 activity (20). Thus, 14-3-3 proteins critically regulate KSR1 function by sequestering it in the cytosol until Ras activation. It is not known whether KSR1 interacts promiscuously with 14-3-3 proteins or, on the contrary, the interaction is isoform-specific. Therefore, we chose KSR1 as a functionally relevant ligand to study 14-3-3 isoform specificity and to gain insight into its own regulation.

EXPERIMENTAL PROCEDURES

Antibodies—Antibodies against KSR1 and 14-3-3 were from BD Biosciences. Antibodies specific for the 14-3-3-3, -γ, -η, -τ and -ζ isoforms, GST, Myc, and an anti-14-3-3 broad antibody were obtained from Santa Cruz Biotechnology. An antibody specific for 14-3-3-ζ was from Lab Vision. The anti-FLAG antibody was from Sigma-Aldrich. Antibodies against Xenopus XMpk1 mitogen-activated protein kinase (MAPK) and Cdc2 have been described (22). An antibody specific for KSR1 phosphorylated on residue Ser-392 was produced by immunizing rabbits with the KLH-conjugated phosphopeptide KSR-pSer-392 (H-LRRTEpSVPSDINC-OH). Phosphospecific antibodies were purified from serum by two-step affinity purification using HiTrap protein A (GE Healthcare) and phosphopeptide-coupled SulfoLink columns (Pierce).

Cell Culture—293 and COS-7 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (HyClone) and antibiotics. U2OS cells, kindly provided by Dr. M. A. Medina (Universidad de Málaga, Spain), were maintained in McCoy’s 5A medium supplemented with 10% fetal bovine serum and antibiotics. Transfections were performed with the FuGENE 6 reagent (Roche Applied Science). Xenopus laevis oocytes were maintained in modified Barth saline (mBarth) medium as described (22).

Cell Lysates—293 or COS-7 cells were washed once with cold PBS and lysed in NP-40 buffer (20 mM Tris-HCl, pH 7.4, 137 mM NaCl, 10% glycerol, 1% Nonidet P-40, 1 mM EDTA, 1 mM dithiothreitol, plus protease and phosphatase inhibitors) (15). Oocyte lysates were prepared by resuspending 3–5 frozen oocytes in 10 μl/oocyte of HIK buffer (80 mM β-glycerophosphate, pH 7.5; 20 mM EGTA, 15 mM MgCl2, 2.5 mM benzamidine, and protease inhibitors) and passing them several times through a micropipette tip. For immunoprecipitation, 250 μg of lysates expressing FLAG-tagged proteins were incubated with 20 μl (50% vol/vol) of FLAG affinity resin (Sigma-Aldrich) for 1 h at 4°C and washed three times with NP-40 buffer. Myc-tagged KSR1 was immunoprecipitated with 2 μg of a polyclonal c-Myc antibody for 2 h at 4°C followed by a 1-h incubation with 30 μl of 50% protein A/G-agarose beads (Santa Cruz Biotechnology).

Plasmids—pCMV-FLAG-KSR1, encoding mouse FLAG-tagged KSR1, was kindly provided by Dr. Richard N. Kolesnick (Memorial Sloan-Kettering Cancer Center, New York). This plasmid was used as a template to obtain the pCMV-FLAG-CA3mut. pCMV-FLAG-KSR1-S297A, pCMV-FLAG-KSR1-S392A, and pCMV-FLAG-KAA plasmids by site-directed mutagenesis with the QuikChange kit (Stratagene). The pCMV-FLAG-KRK/KSR1 plasmid was constructed exactly as described (23). The seven isoforms of 14-3-3 proteins (β, γ, ε, η, σ, τ, and ζ), each in a pGEX-6P1 vector, were provided by Dr. Cheryl L. Walker (Memorial Sloan-Kettering Cancer Center, Houston, TX). These constructs were used to generate their Myc-tagged counterparts for expression in mammalian cells as follows. The β, γ, η, σ, and τ isoforms were subcloned into the BamHI and EcoRI sites of pCMV-Tag3B (Stratagene), and the ε and γ isoforms were subcloned into the BamHI and XhoI sites. The 14-3-3ζ cDNA was amplified by PCR using the pGEX-6P1-14-3-3ζ plasmid as template and primers zBamHI and zEcoRI (the sequences of all primers used in this study can be found in supplemental Table 1). The PCR product was digested with BamHI/EcoRI and ligated into pCMV-Tag3B. The plasmid pCMV-FLAG-14-3-3γ was obtained by digestion of pCMV-myc-14-3-3γ with BamHI/XhoI and ligation of the resulting fragment into pCMV-Tag2B (Stratagene). To obtain the plasmids for the mammalian two-hybrid experiments, mouse KSR1 was amplified by PCR using pCMV-FLAG-KSR1 as template and primers KSR6b and KSR17. The PCR product was digested with EcoRI/XbaI and subcloned into the plM vector (Stratagene). Primers MycBgIII and pCMV3-XbaI were used to amplify the cDNAs coding for the seven 14-3-3 isoforms, using the Myc-tagged constructs as templates. The PCR products
were digested with BglII/SalI and ligated into pFTX4. Constructs FTX4-14-3-3ε, pCMV-myc-14-3-3γ, and pCMV-myc-14-3-3ζ, respectively, to amplify nucleotides coding for residues 1–235 of 14-3-3γ, 1–236 of 14-3-3ε, and 1–236 of 14-3-3ζ. The PCR fragments were digested with NotI/EcoRV and ligated into pCMV-Tag3B to generate plasmids pCMV-myc-14-3-3γC, pCMV-myc-14-3-3εC, and pCMV-myc-14-3-3ζC. The 14-3-3γ–ε isoforms plus vector sequence downstream of the multicloning site were amplified with forward primers γ‘5’-EcoRV, ε‘5’-EcoRV, γ5’-SmaI, ε5’-SmaI, and the common reverse primer pCMV3‘-DraIII. Finally, the PCR products were digested with BglII/SalI and ligated into pFTX5 precut with BamHI/EcoRI and ligated into pFTX4. Vectors pFTX4 and pFTX5 have been described (22, 24). The pCMV-myc-14-3-3γ-K50E mutant was obtained with mutagenic primers 1433yK50EF and 1433yK50Er. The 1433yS59DF and 1433yS59Dr primers were used to clone the mutant pCMV-myc-14-3-3γS59D and pCMV-FLAG-14-3-3γS59D constructs. The EYFP-difopein and EYFP-R18(Lys) plasmids were gifts from Dr. Haian Fu (Emory University, Atlanta, GA).

**Mammalian Two-hybrid Assay**—293 cells were seeded in 60-mm plates (500,000 cells/plate) and transfected with a combination of the following plasmids: pM-CAT reporter (0.2 μg), pM-KSR1 (1.5 μg), and pVP16-myc-14-3-3 specific for each isoform (0.5 μg for γ, 1.0 μg for η, ζ, and σ, and 2.0 μg for β, ε, and ζ). Forty-eight hours later, CAT expression, indicative of the in vivo KSR1/14-3-3 interaction, was measured by a colorimetric enzyme immunoassay using the CAT ELISA kit (Roche Applied Science).

**Oocyte Meiotic Maturation Assay**—X. laevis oocytes were prepared essentially as described (22). Plasmids pFTX5-KSR1 and pFTX4-14-3-3 (2–3 μg) were linearized and used as tem-
plates for capped mRNA synthesis with the mMESSAGE mACHINE in vitro transcription kit (Ambion Inc.). To avoid inhibition of Ras-induced maturation due to KSR1 scaffolding activity (17), the amount of injected KSR1 mRNA was titrated previously. Oocytes were microinjected with 50 nl of the in vitro transcribed mRNAs (2.5 ng for Myc-KSR1 and 35 ng for HA-14-3-3) and maintained at 18 °C in mBarth medium. Five hours later the oocytes were reinfected with 50 ng of a mRNA encoding Myc-H-RasG12K and scored for germinal vesicle breakdown (GVBD) as a measure of meiotic maturation. GVBD was scored when 5–10% of the oocytes injected with Ras alone had undergone GVBD (usually 6–8 h after injection) (17).

RNA Interference—Synthetic siRNAs specific for human 14-3-3-γ (NM_012479) or 14-3-3-τ (NM_006826) were purchased from Ambion Inc. as 19-mer complementary RNA duplexes with UU overhangs at their 3’-ends. A scrambled sequence with no homology in the human genomic data base was used as a negative control. U2OS cells were seeded in 6-well plates and transfected 24 h later (day 1) with the corresponding siRNAs (20 nM) by the calcium phosphate method. The cells were retransfected 24 h later (day 2) following the same protocol, and on day 3, the cells were transfected with 2.0 μg of pCMV-FLAG-KSR1 using the FuGENE 6 reagent. On day 5, the cells were processed for Western blot and immunofluorescence.

Immunofluorescence—Transfected cells grown on coverslips in 6-well plates were formalin-fixed, permeabilized with PBS plus 0.2% Triton X-100, and stained either singly or doubly with a mouse monoclonal FLAG antibody and a rabbit polyclonal Myc antibody followed by an Alexa 488-conjugated goat anti-rabbit antibody and a rhodamine-conjugated goat anti-mouse antibody.

Isolation of 14-3-3 Dimers Bound to KSR1—Total cell lysates (3.5 mg) prepared from 293 cells coexpressing FLAG-KSR1 and Myc-14-3-3-γ were immunoprecipitated with anti-FLAG beads as described above and washed sequentially with NP-40 buffer (five times) and Tris-buffered saline (twice). Bound FLAG-KSR1 was eluted twice by incubation at 15 °C for 15 min in 35 μl of 0.1 mg/ml FLAG peptide (Sigma-Aldrich) diluted in Tris-buffered saline. The combined eluates were diluted 1:2 in NP-40 buffer. One-half was immunoprecipitated with 5.6 μg of a Myc polyclonal antibody, and the other half was immunoprecipitated with 5.6 μg of a non-immune rabbit IgG for 2 h at 4 °C followed by a 1-h incubation with 30 μl of protein A-Sepharose. Immunoprecipitates were washed three times with NP-40 buffer, resuspended in 70 μl of Laemmli’s sample buffer, and fractionated by SDS-PAGE followed by Western blot with specific anti-14-3-3 antibodies. A 10 μl-aliquot was loaded per well. As a control, we also immunoprecipitated sequentially, with FLAG and Myc antibodies, a 293 lysate expressing FLAG-KSR1 alone.

Cross-linking Experiments—COS-7 lysates expressing either FLAG-14-3-3-γ or FLAG-14-3-3-τ-S59D were subjected to immunoprecipitation with an anti-FLAG affinity resin. Beads were washed three times with NP-40 buffer and twice with phosphate buffer. Dimers in the immunoprecipitates were chemically cross-linked with 50 μg/ml bis(sulfosuccinimidyl) suberate (Sigma-Aldrich) as described (27).

Molecular Modeling—Interactions of KSR1 phosphopeptides with residues in the 14-3-3-γ basic pocket were modeled with the program Mutmodel (28) using the crystal structure of human 14-3-3-γ bound to mode 1 phosphopeptide RAlpSPLP (Protein Data Bank ID: 2B05) as template. Water accessibility for 14-3-3-γ protein was calculated with the DSPP program (29). The DALI program (30) was used to superimpose the different 14-3-3 proteins complexes retrieved from the Protein Data Bank and the two modeled KSR1 phosphopeptides, using the 2B05 coordinates as the fixed structural reference. The three-dimensional images were rendered with VMD (Visual Molecular Dynamics) (30).

RESULTS

KSR1 Interacts Preferentially with 14-3-3-γ in Vitro—The seven human 14-3-3 isoforms are highly similar at the amino acid level, sharing an average identity of 70% and having strictly conserved residues directly involved in pSer recognition (9). To gain insight into the functional role of 14-3-3 proteins in KSR1 regulation, we first searched for any isotype-related differences in binding. The seven GST-tagged human 14-3-3 isoforms were purified to near homogeneity (supplemental Fig. 1A) and used as baits in pulldown experiments with FLAG-KSR1-expressing cell lysates. We could not perform direct interaction assays because production of full-length recombinant KSR1 was not possible in our laboratory. This problem has also been reported by other groups (31). As shown in Fig. 1, A and B, no KSR1 bound to recombinant 14-3-3-τ, whereas only the highest concentrations of the ε and τ isoforms were able to poorly associate with KSR1. On the contrary, KSR1 was efficiently pulled down, albeit to different extents, by isoforms β, γ, η, and ζ. At any concentration tested, 14-3-3-γ consistently showed the strongest interaction with KSR1, suggesting a higher affinity of this isoform for KSR1. These differences cannot be attributed to altered binding properties of the GST fusion proteins, because difopein, a specific 14-3-3 ligand that does not possess isoform selectivity (32), was pulled down in similar amounts by all GST-14-3-3 proteins (supplemental Fig. 1B). In addition, none of them bound to the mutant ligand R18(Lys), indicating that the interaction occurred within the amphipathic groove (supplemental Fig. 1B) (32).

A combined pulldown/IP depletion assay confirmed that the amount of FLAG-KSR1 competent for binding to 14-3-3 (i.e. KSR1 phosphorylated on Ser-392) was not limiting in these assays (supplemental Fig. 1, C and D).

KSR1 forms a multiprotein complex inside the cells (18). Therefore, a legitimate caveat can be added that the interaction observed between KSR1 and 14-3-3-γ is mediated through a KSR1-associated protein and not by KSR1 itself. To exclude that possibility, we performed pulldown experiments with lysates expressing FLAG-KAA, a KSR1 mutant lacking the two consensus 14-3-3-binding sites, Ser-297 and Ser-392 (17). We did not detect binding of FLAG-KAA to GST-14-3-3γ at any of the amounts tested (Fig. 1A). Next, we performed competition experiments with synthetic phosphopeptides based on amino acid sequences surrounding KSR1 residues Ser-297 and Ser-
14-3-3 Isoform-specific Binding to KSR1

A  GST Pull-down

GST-14-3-3

KSR1

14-3-3β

14-3-3γ

14-3-3ε

14-3-3ζ

14-3-3η

14-3-3ω

14-3-3τ

B  GST Pull-down

GST-14-3-3

KSR1

14-3-3β

14-3-3γ

14-3-3ε

14-3-3ζ

14-3-3η

14-3-3ω

14-3-3τ

C  Competing Peptides

GST-14-3-3γ

GST

PS-297: H-TLTRSKpSHESQQL-OH

PS-392: H-LRRTEPVSVPDIN-OH

Buffer

P-297

P-297

P-392

P-392

D  GST Pull-down

B-L

GST

GST-14-3-3

B-KSR1

β

ε

η

σ

τ

ζ

FIGURE 1. 14-3-3/KSR1 interaction in vitro. A, increasing amounts of GST-14-3-3 isoforms were incubated with 250 μg of a 293 cell lysate expressing FLAG-KSR1. The amount of pulled down FLAG-KSR1 in each sample was determined by WB with a FLAG antibody, and GST-14-3-3 loading was controlled by Coomassie staining. As a control, pulldowns were done with GST-14-3-3γ and lyses expressing FLAG-KAA, a KSR1 mutant that cannot bind 14-3-3. Shown are blots representative of the results obtained in four independent experiments. B, quantification of the results shown in A (mean ± S.D. of three independent experiments). C, pulldown assays were performed in the absence (Buffer) or presence (150 μl) of the competing PS-297 and PS-392 phosphopeptides or their unphosphorylated equivalents (P-297; P-392). D, pulldown assays were performed with 10 μg of GST-14-3-3 proteins and 1.5 mg of a mouse brain lysate (B.L.). The intensity of the B-KSR1 bands, as detected with a KSR1 antibody, was quantitated by densitometry and normalized to the value for γ = 1.0.

Preincubation of GST-14-3-3γ with phosphopeptides PS-297 and PS-392, alone or in combination, provoked a marked reduction in KSR1 binding, whereas their nonphosphorylated counterparts had no effect (Fig. 1C). Collectively, these results confirm that the observed interaction between KSR1 and 14-3-3γ is specific and is mediated by direct interaction with pSer-297 and pSer-392 in KSR1.

To allow for binding measurements at lower concentrations of the bait protein, we adapted the pulldown assay to a microtiter plate (ELISA) format. Again, 14-3-3γ exhibited the highest affinity for KSR1 followed by the η, β, and ζ isoforms (supplemental Fig. 1E). At the low concentrations tested in the assay, we did not detect binding to the ε, σ, or τ isoform nor did we observe binding of 14-3-3γ to the KAA mutant (data not shown).

The different affinities observed in the above experiments were not an artifact of overexpression of recombinant FLAG-KSR1, as pulldown experiments done with mouse brain lysates showed a similar pattern of interaction. Thus, endogenous B-KSR1 (33) bound preferentially to 14-3-3γ and 14-3-3η, whereas lower amounts bound to the β, τ, and ζ isoforms (Fig. 1D). B-KSR1 did not interact with 14-3-3ε or 14-3-3ω. In summary, in vitro assays demonstrated a marked substrate discrimination among different 14-3-3 isoforms with 14-3-3γ being particularly selective for KSR1.

KSR1 Interacts Preferentially with 14-3-3γ in Vivo—To determine whether KSR1 also interacts preferentially with 14-3-3γ in vivo, we performed communoprecipitation (co-IP) experiments with lyses expressing FLAG-KSR1 and each of the seven Myc-tagged human 14-3-3 isoforms. Only 14-3-3γ and 14-3-3η were detected in the FLAG immunoprecipitates (Fig. 2A). In contrast, all seven isoforms associated similarly with EYFP-difopein, indicating that the Myc-tagged proteins are fully functional (supplemental Fig. 2A). Consistent with the results shown in Fig. 1, the amount of 14-3-3γ associated with KSR1 was higher than that of 14-3-3η, strongly confirming an intrinsic higher affinity of the γ isoform for KSR1. Using twice the amount of lysate gave the same result (data not shown). A 14-3-3γ protein with a K50E mutation, which reportedly impairs ligand binding (34), did not associate with KSR1 (Fig. 2B), indicating that the interaction inside the cell is mediated by the phosphate binding cleft. To further control for the specificity of the experiment we performed co-IP studies with C-Raf, which is the closest relative to KSR1 and displays overall sequence similarity and domain organization (13). FLAG-C-Raf immunocomplexes contained detectable levels of the γ, η, ε, τ, and ζ isoforms, suggesting a more promiscuous interaction with 14-3-3 (Fig. 2C). In contrast to FLAG-KSR1, we consistently observed similar levels of the γ and η isoforms bound to FLAG-C-Raf (Fig. 2C).

KSR1 and C-Raf share structurally related cysteine-rich domains (supplemental Fig. 2B) (23). Because structure-disrupting mutations in both CRDs have been reported to reduce 14-3-3 binding (17, 35), we wanted to determine whether the...
CRD of KSR1 (named CA3) confers specificity to its interaction with 14-3-3γ. A KRK chimeric mutant was generated by swapping CA3 with the CRD domain of C-Raf. Co-IP experiments showed that, similar to KSR1, the KRK mutant only interacted with the γ and η isoforms (supplemental Fig. 2C). No significant differences were observed in the binding of 14-3-3γ to KSR1 or KRK as determined by co-IP (supplemental Fig. 2C, compare lanes 1 and 4) or in our ELISA-based assay (supplemental Fig. 2D). On the contrary, a KSR1 mutant lacking a functional CA3 domain (CA3mut) was greatly impaired in its ability to interact with 14-3-3γ (supplemental Fig. 2D). Taken together, these results suggest that the overall three-dimensional structure of CRDs may play a role in 14-3-3 substrate recognition, whereas variations in the sequence of different CRDs seem to be less relevant.

Next, we transiently transfected COS-7 cells, as shown in Fig. 2A, and examined them by indirect immunofluorescence. KSR1 colocalized almost completely with 14-3-3γ and 14-3-3η and partially with 14-3-3β (supplemental Fig. 2F). Little colocalization with KSR1 was observed for the ε, σ, τ, and ζ isoforms and for 14-3-3γ with the AA mutant.

Finally, we used a mammalian two-hybrid system to quantitate the in vivo binding of each isoform to KSR1. Cotransfection of KSR1 and 14-3-3γ induced the highest expression of CAT protein, indicative of a strong interaction between both proteins (Fig. 2D). CAT expression correlated well with the different affinits observed for each isoform in the co-IP assays, with 14-3-3γ being the second preferred isoform after 14-3-3γ. The fact that some CAT was produced by coexpression of KSR1 and 14-3-3σ, which did not bind KSR1 in any of our assays, might be technically related; the two-hybrid system is designed so that expressed proteins are directed to the nucleus, where we cannot exclude a KSR1/14-3-3σ association of unknown relevance. In sum, the above experiments confirmed in vivo the existence of ligand specificity among 14-3-3 isotypes and, in particular, that of 14-3-3γ for KSR1.

Functional Relevance of the 14-3-3γ/KSR1 Interaction—Next, we wanted to determine whether the 14-3-3γ/KSR1 interaction was affected by growth factors. To that end, 293 cells cotransfected with both proteins were stimulated with EGF, and the amount of 14-3-3γ bound to KSR1 was determined in co-IP experiments. In agreement with the current model of KSR1 activation (19), a 5-min EGF stimulation reduced the 14-3-3γ/KSR1 interaction concomitant with a dephosphorylation of KSR1 in residue Ser-392 (Fig. 3A), likely due to activation of the phosphatase PP2A. This effect was transient, as KSR1 and 14-3-3γ began to reassociate 30–60 min after stimulation, coincident with an increase in pSer-392 levels (Fig. 3A). Next, we coexpressed KSR1 with increasing amounts of 14-3-3γ and determined the amount of pSer-392 after EGF

FIGURE 2. 14-3-3/KSR1 interaction in vivo. A, co-IP assays were performed in COS-7 lysates (250 μg) expressing the indicated constructs using a FLAG resin. Expression of exogenous proteins was determined by WB (Total Lysate). B, co-IP was performed with lysates expressing FLAG-KSR1 together with Myc-14-3-3γ (γ) or Myc-14-3-3γ-K50E (γ-K50E), a mutant deficient for ligand binding. C, co-IP assays were done as in A except that lysates expressed FLAG-C-Raf instead of FLAG-KSR1. D, 293 cells were transfected with a CAT reporter plasmid together with KSR1 and the seven Myc-14-3-3 isoforms. Lysates were prepared 48 h later, and the levels of CAT protein, indicative of a 14-3-3/KSR1 interaction, were determined. Values were normalized to the amount of CAT produced by KSR1 alone and expressed as the percentage of CAT produced in the cells coexpressing KSR1 and 14-3-3γ. Three independent experiments were performed in triplicate (mean ± S.D.). Appropriate expression of the proteins in total lysates is shown in representative blots.
14-3-3 Isoform-specific Binding to KSR1

A

![Graph showing percentage of FLAG-positive cells transfected with S, FLAG-KSR1, myc-14-3-3γ, and EGF](image)

B

![Graph showing percent GVBD with FLAG-KSR1(pS392), EGF, Flag-KSR1, and myc-14-3-3γ](image)

C

![Bar graph showing percent GVBD with myc-RasG12K, myc-KSR1, HA-14-3-3s, myc-RasG12K, myc-KSR1, HA-14-3-3, ERK2, and CDK1](image)

D

![Diagram showing siRNA targeting 14-3-3τ, S, γ, or τ and its effect on plasma membrane localization](image)

FIGURE 3. Functional significance of the 14-3-3γ/KSR1 interaction. A, 293 quiescent cells, expressing the indicated proteins, were stimulated at different times with 50 ng/ml EGF, and co-IP experiments were performed as described for Fig. 2A. The activation state of KSR1 was determined by WB with an antibody specific for KSR1pS392. B, cells were transfected with KSR1 alone or in combination with increasing amounts of 14-3-3γ, stimulated for 5 min with EGF, and processed for co-IP. Quantification of KSR1(pS392) normalized to total KSR1 in the immunoprecipitates is shown. C, oocytes were microinjected with mRNAs encoding for Myc-KSR1 and each of the seven HA-14-3-3 isoforms. They were reinjected 5 h later with a mRNA specific for Myc-RasG12K. GVBD was scored when 5–10% of the oocytes injected with Myc-RasG12K alone had initiated maturation. Translation of the mRNAs was assessed by WB with Myc and HA antibodies. Activation of ERK2 was detected by WB as a decrease in its electrophoretic mobility (due to phosphorylation), whereas activation of CDK1 resulted in a faster migrating band (due to dephosphorylation). D, U2OS cells grown in 6-well plates with or without coverslips were mock-transfected (C, control) or transfected twice with 20 nM siRNAs either scramble (S) or specific for 14-3-3τ (τ) or 14-3-3γ (γ). On day 3, cells were transfected with FLAG-KSR1. Endogenous levels of 14-3-3γ, 14-3-3τ, and actin were determined on day 5 by WB with specific antibodies (upper panel). Cells grown on coverslips were processed for immunofluorescence with a FLAG antibody (lower panel) to detect membrane localization of FLAG-KSR1 (arrows). Selected areas of plasma membrane (bracketed) are shown enlarged. The graph shows the percentage of FLAG-positive cells transfected with S, γ, or τ siRNAs showing membrane localization (mean ± S.D.). At least 250 cells/condition were counted in two independent assays.

addition. Stimulation of quiescent cells expressing FLAG-KSR1 alone provoked a reproducible dephosphorylation of pSer-392 (Fig. 3B). However, expression of Myc-14-3-3γ inhibited dephosphorylation of KSR1 in a dose-dependent manner (Fig. 3B) indicating that 14-3-3γ can regulate KSR1 signaling by protecting it from PP2A-mediated dephosphorylation (which likely explains the relatively modest effect of EGF on pSer-392 observed in Fig. 3A). These results demonstrate that KSR1 and 14-3-3γ form functional complexes that are responsive to growth factor stimulation.

KSR1 has been shown to facilitate oncogenic Ras signaling in Xenopus oocytes (17) and 14-3-3 proteins play an essential role in that process by sequestering KSR1 in the cytosol until Ras activation (19, 21). We reasoned that KSR1 would fail to cooperate with Ras in oocytes overexpressing KSR1-interacting 14-3-3 isoforms because of its enhanced cytoplasmic retention. On the contrary, overexpression of those isoforms that do not interact with KSR1 should not interfere that function. KSR1 interacted with the same 14-3-3 isoforms in oocytes and in mammalian cells (supplemental Fig. 3A). We microinjected oocytes with mRNAs encoding Myc-KSR1 and HA-14-3-3 isoforms together with oncogenic RasG12K and scored the percentage of oocytes undergoing maturation (% GVBD). Overexpression of 14-3-3γ or 14-3-3η completely blocked the ability of
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To further confirm the functional relevance of the 14-3-3/ KSR1 interaction, we next inhibited the expression of endogenous 14-3-3γ and 14-3-3τ in human U2OS cells by using specific siRNAs and looked at the subcellular distribution of FLAG-KSR1. We were not able to silence expression of the β, ε, η, σ, and ζ isoforms by using either synthetic siRNAs or short hairpin RNA-expressing vectors (data not shown). The γ and τ isoforms were reduced by 60% at the protein level (Fig. 3D, upper panel). Silencing of endogenous 14-3-3γ provoked a marked accumulation of FLAG-KSR1 at the plasma membrane, whereas silencing of 14-3-3τ had no effect (Fig. 3D, lower panel). These results, as well as those from Fig. 3C, are in agreement with a specific role for 14-3-3γ in preventing uninduced KSR1 translocation to the plasma membrane.

Molecular Modeling of the 14-3-3γ/KSR1 Interaction—To search for a structural basis underlying the specific binding of 14-3-3γ to KSR1, we modeled the interaction of KSR1 phosphopeptides RSKP SHE (PS297) and RTEpSVP (PS392) with 14-3-3γ using the published structure of 14-3-3γ bound to a mode 1 phosphopeptide, RAIpSLP, as template (9). The resulting models (Fig. 4A and B) showed that all 14-3-3γ residues predicted to have at least 1 atom root mean square distance <5.0 Å from any atom in PS297 or PS392 are strictly conserved in the seven human isoforms, including residues Lys-50, Lys-57, Arg-132, and Tyr-133, which hold the pSer phosphate in all solved structures (9) (data not shown). These results suggest that interaction conformation specificity is determined mainly by the phosphopeptide sequence rather than by residues in the binding groove. In fact, superimposing the same 14-3-3 isoform bound to different phosphopeptides revealed changes in their spatial conformation, particularly toward their N and C termini (data not shown). The conserved Arg at pSer-3 showed a broad range of conformational freedom, making any conformation prediction at the N terminus too speculative. Interestingly, however, our model revealed that phosphopeptide structures with polar instead of hydrophobic residues in pSer+1 show a significant shift in the orientation of the conserved Pro in pSer+2. The predicted conformation and orientation of PS392 at the C terminus was similar to those in phosphopeptides with hydrophobic C-terminal sides (Fig. 4C). In marked contrast to PS392, PS297 has a highly polar C-terminal side (pSHE) and lacks the conserved Pro. As a result, PS297 cannot complement the conserved 14-3-3 hydrophobic patch (36) and is predicted by our model to be shifted away from the binding cleft (Fig. 4D), which might explain its reported lower binding affinity for 14-3-3γ (17). In summary, our model indicates that direct side-chain interactions at the conserved ligand binding cleft do not seem to account for 14-3-3γ specific binding to KSR1, whereas residue composition at the C terminus of its two consensus binding sites might determine their binding affinity.

The C Terminus of 14-3-3γ Participates in Selective Binding to KSR1—A phylogenetic tree of the human 14-3-3 proteins shows that the closest isoforms to the related γ and η are the β, ζ, and τ, whereas the ε and σ are classified in different clades (37). Interestingly, this ranking correlates fairly well with the different isoform affinities observed for KSR1 (Figs. 1 and 2). The highest level of variation among isoforms occurs in a C-terminal stretch (CTS) of acidic residues. Particularly, 14-3-3y and 14-3-3τ share similar short CTSs, while the ε and σ isoforms have the longest and most divergent CTSs (Fig. 5A). To investigate whether this region confers specificity to the 14-3-3γ/ KSR1 interaction, we deleted the CTS in each 14-3-3 isoform
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A

hEta (215–246) hGamma (215–247) hBeta (212–246) hZeta (210–245) hTau (210–245) hSigma (212–248) hEpsilon (213–255)

B

Flag-KSR1 +

IP: α-Flag

myc-14-3-3s Flag-KSR1 myc-14-3-3

Total Lysate

Flag-KSR1 myc-14-3-3

myc-14-3-3

C

Flag-KSR1 +

IP: α-Flag

myc-14-3-3s Flag-KSR1 myc-14-3-3

Total Lysate

Flag-KSR1 myc-14-3-3

myc-14-3-3

D

Flag-KSR1 +

IP: α-Flag

myc-14-3-3s Flag-KSR1 myc-14-3-3

Total Lysate

Flag-KSR1 myc-14-3-3

myc-14-3-3

E

Flag-KSR1 +

IP: α-Flag

myc-14-3-3s Flag-KSR1 myc-14-3-3

Total Lysate

Flag-KSR1 myc-14-3-3

myc-14-3-3

FIGURE 5. The CTS of 14-3-3γ participates in KSR1 recognition. A, The C termini of the seven human 14-3-3 isoforms (residues shown in parentheses) were first aligned using a pairwise-based software and then manually according to the length of their divergent CTSs. White characters denote strict identity, and bold characters denote similarity within a group. Similarity across groups is highlighted by frames. B, Myc-14-3-3 deletion mutants (ΔC), each lacking a specific CTS, were coexpressed in COS-7 cells with FLAG-KSR1, and their interaction was determined by co-IP as described in Fig. 2A, C, binding of γ-ΔC and η-ΔC mutants to KSR1 was compared with that of their wild-type counterparts by co-IP, D, the C-terminal tails of 14-3-3γ and 14-3-3ε were swapped to generate the chimeric constructs 14-3-3γε and 14-3-3εγ, and their binding to KSR1 was assessed by co-IP, E, the relevance of the CTS sequence for KSR1 binding to 14-3-3γ and 14-3-3η was determined in co-IP assays using COS-7 cells lysates expressing FLAG-KSR1 together with the indicated 14-3-3γ and 14-3-3η chimeric mutants.

(ΔC mutants) and determined the ability of the remaining protein core to interact with KSR1 in co-IP experiments. The ΔC mutants behave identically to full-length proteins, with the γ and η isoforms being the only ones bound to KSR1 (Fig. 5B), which indicates that isoform specificity is dictated mainly by the protein core. However, 14-3-3γ-ΔC and 14-3-3η-ΔC mutants consistently showed a 70–80% reduction in binding to KSR1 when compared with their wild-type counterparts (Fig. 5C), suggesting a critical role for their CTSs in binding to KSR1. Next, we investigated whether the CTS plays a general role in ligand binding or, on the contrary, its sequence is relevant for ligand discrimination. To that end, we generated chimeric 14-3-3 molecules by swapping the CTSs of 14-3-3γ and 14-3-3ε. Compared with wild-type 14-3-3γ, the 14-3-3γε chimera bound KSR1 less efficiently (Fig. 5D), to an extent similar to that shown by the 14-3-3γ-ΔC mutant (Fig. 5E), indicating that a CTS highly divergent from its own cannot confer full ligand binding to 14-3-3γ. On the other hand, adding the CTS of 14-3-3γ to 14-3-3ε-ΔC did not increase its ability to interact with KSR1 (Fig. 5D). Interestingly, a 14-3-3γ/ζ chimera bound KSR1 only slightly better than 14-3-3γ/ε, and a 14-3-3γ/η chimera bound KSR1 to an extent similar to 14-3-3γ (Fig. 5E). These results indicate that the similar CTSs of 14-3-3γ and 14-3-3η contribute to the preferred interaction of these isoforms with KSR1. Further supporting this notion, adding the CTS of 14-3-3γ to 14-3-3η-ΔC (14-3-3η/γ mutant) restored binding to KSR1 (Fig. 5E).

Homo- and Heterodimeric Forms of 14-3-3γ Are Bound to KSR1—We next evaluated the role played by dimerization in 14-3-3γ ligand discrimination. The Myc-14-3-3γ protein used in our studies was essentially able to homo- and heterodimerize with all overexpressed isoforms except 14-3-3ζ (Fig. 6A). Myc-14-3-3γ also formed heterodimers with all endogenous isoforms except ε and η (Fig. 6B). Therefore, the possibility exists that some of the interactions observed in our experiments were reflecting selective 14-3-3γ heterodimers. To test this hypothesis, we developed an experimental protocol to specifically isolate the KSR1-bound pool of 14-3-3γ molecules and determine
its dimerization profile with other 14-3-3 isoforms (Fig. 6C). We detected γ/γ homodimers and γ/τ and γ/ε heterodimers associated with KSR1 (Fig. 6D). We did not detect the ε and σ isoforms as part of the 14-3-3γ/KSR1 complex (Fig. 6D), in agreement with their lack of interaction or functional effect on KSR1 observed in different assays (Figs. 1–3). The absence of 14-3-3σ in the complex was also expected, as this isoform preferentially forms homodimers (37). Interestingly, 14-3-3η and 14-3-3β were also absent from the 14-3-3γ/KSR1 complex (Fig. 6D), suggesting that the interaction observed in our binding assays reflects a direct and specific binding of these isoforms to KSR1. In the case of 14-3-3η, we cannot exclude an interaction below the detection limit of the antibody because the signal in the control lysate was typically weak (Fig. 6D). The 14-3-3 antibodies used were fairly specific, with the exception of the anti-14-3-3η, which cross-reacts with the γ and ε isoforms (supplemental Fig. 3B). This nonspecificity lacks relevance here, as no WB signal could be detected in the 14-3-3γ/KSR1 complex using that antibody (Fig. 6D). Thus, 14-3-3γ binds to KSR1 as a homodimer or as a heterodimer with selected isoforms.

**Role of 14-3-3γ Dimerization**—To further elucidate the role of 14-3-3γ dimerization in KSR1 binding, we attempted to generate a 14-3-3γ dimer-deficient mutant. Phosphorylation of 14-3-3γ on Ser-58 or its phospho-mimic mutation to either Asp or Glu severely impairs dimer formation due to an increase in observed with the 14-3-3γ-Δσ mutant (Fig. 5E), suggesting that the CTS of 14-3-3γ plays a more critical role than its dimerization status with regard to KSR1 interaction. To determine the KSR1 binding site for monomeric 14-3-3γ, we coexpressed 14-3-3γ-S59D with KSR1 mutants lacking one (S297A and S392A) or two (KAA) residues involved in 14-3-3 binding. As shown in Fig. 7C, elimination of any of the two serines reduced binding to monomeric 14-3-3γ. Nevertheless, the S392A mutant was still able to support interaction with 14-3-3γ-S59D, indicating that the remaining site, Ser-59, was competent for binding to monomeric 14-3-3γ (Fig. 7C, long exposure).

**DISCUSSION**

The identification of 14-3-3 isoform-specific ligands is of cardinal importance in the field of signal transduction, as it might help to discriminate functional roles in this highly conserved family of proteins (1). 14-3-3 proteins have evolved so that unicellular eukaryotes have only a few isoforms, whereas multicellular ones have many (39), likely reflecting the need for isoform-specific functions in complex organisms. Here, we have reported a detailed analysis, both in vitro and in vivo, of functional specificity in 14-3-3 proteins, including all human isoforms. In a variety of experimental settings, the molecular scaffold KSR1 consistently showed a marked preference for binding to 14-3-3γ, although it was able also to interact with...
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Some but not all 14-3-3 isoforms (\(\eta > \beta_\xi > \gamma\)). In vivo KSR1 interacted almost exclusively with 14-3-3\(\gamma\) and 14-3-3\(\eta\) (\(\gamma > \eta\)). Notably, this ligand specificity correlated with sequence similarity among the seven human isoforms, which, given their conserved structural features, strongly suggests that regions of sequence variation among 14-3-3 isoforms contribute to ligand discrimination. In particular, the short stretch of acidic residues (CTS) located at their C termini is the most divergent region among isoforms, and we have demonstrated here its critical role in KSR1 recognition. The in vitro assays showed that the longer the CTS, the weaker the binding to KSR1. Also, KSR1 associated in vivo exclusively with 14-3-3\(\gamma\) and 14-3-3\(\eta\), which share a short and almost identical CTS. Therefore, both the sequence and the length of the CTS seem to be important for substrate specificity. Previous work from Truong et al. (40) had shown that a \(\Delta C\) form of 14-3-3\(\zeta\), lacking its CTS, could bind to Raf1 and BAD with higher affinity than the full-length protein, supporting the notion of an autoinhibitory role for that region. These data could reflect a specific feature of 14-3-3\(\zeta\) binding to Raf1 and BAD or be due to alleviation of the reported phosphorylation-induced conformational change in the 14-3-3\(\zeta\) CTS, which inhibits ligand binding (41). In our hands, however, deletion of the CTS impaired KSR1 binding to the \(\gamma\) and \(\eta\) isoforms and did not increase binding to the other isoforms. Notably, the CTSs of these two related isoform were interchangeable, whereas swapping the CTSs between two dissimilar isoforms like 14-3-3\(\gamma\) and 14-3-3\(\xi\) did not allow full binding to KSR1. A 14-3-3\(\gamma\)/\(\zeta\) chimera was intermediate between 14-3-3\(\gamma\)/\(\xi\) and 14-3-3\(\gamma\)/\(\eta\) in terms of KSR1 binding, further indicating that sequence similarity at the CTS also correlates with isoform specificity. According to Obsil and co-workers (42), the flexible CTS folds back into the ligand binding pocket and can be displaced only by specific phosphopeptide sequences. Our results are compatible with this model, although, rather than a strictly autoinhibitory role, they suggest a positive role for the CTS in ligand discrimination by stabilizing specific interactions engaged by the core of the 14-3-3 protein. They are also in accordance with recent data from optimal docking area studies suggesting that, secondary to the phosphopeptide binding, general protein-protein interactions at the so-called “desolvation patches” and subsequent chain/loop rearrangements allow for isoform-specific ligand binding (36). In sum, our results suggest that contacts with the core of each 14-3-3 isoform mainly determine substrate specificity. Once the primary interaction has occurred, the isoform-specific CTSs seems to recognize bona fide ligands and stabilize the complex.

FIGURE 7. Role of 14-3-3\(\gamma\) dimerization in KSR1 binding. A, immunoprecipitates of FLAG-14-3-3\(\gamma\) or FLAG-14-3-3\(\gamma\)-S59D were treated or not with the chemical cross-linker bis(sulfosuccinimidy) suberate (BS\(\gamma\)), fractionated by SDS-PAGE, and immunoblotted with a 14-3-3\(\gamma\) antibody to reveal its monomeric and dimeric forms. An aliquot of the lysate shows the endogenous and the FLAG-tagged 14-3-3\(\gamma\) proteins. B, FLAG-14-3-3\(\gamma\) and FLAG-14-3-3\(\gamma\)-S59D were coexpressed with the seven Myc-tagged human 14-3-3 isoforms to determine their interaction almost exclusively with 14-3-3\(\gamma\) and 14-3-3\(\alpha\), which, given their conserved structural features, strongly suggests that regions of sequence variation among 14-3-3 isoforms contribute to ligand discrimination. In particular, the short stretch of acidic residues (CTS) located at their C termini is the most divergent region among isoforms, and we have demonstrated here its critical role in KSR1 recognition. The in vitro assays showed that the longer the CTS, the weaker the binding to KSR1. Also, KSR1 associated in vivo exclusively with 14-3-3\(\gamma\) and 14-3-3\(\eta\), which share a short and almost identical CTS. Therefore, both the sequence and the length of the CTS seem to be important for substrate specificity. Previous work from Truong et al. (40) had shown that a \(\Delta C\) form of 14-3-3\(\zeta\), lacking its CTS, could bind to Raf1 and BAD with higher affinity than the full-length protein, supporting the notion of an autoinhibitory role for that region. These data could reflect a specific feature of 14-3-3\(\zeta\) binding to Raf1 and BAD or be due to alleviation of the reported phosphorylation-induced conformational change in the 14-3-3\(\zeta\) CTS, which inhibits ligand binding (41). In our hands, however, deletion of the CTS impaired KSR1 binding to the \(\gamma\) and \(\eta\) isoforms and did not increase binding to the other isoforms. Notably, the CTSs of these two related isoform were interchangeable, whereas swapping the CTSs between two dissimilar isoforms like 14-3-3\(\gamma\) and 14-3-3\(\xi\) did not allow full binding to KSR1. A 14-3-3\(\gamma\)/\(\zeta\) chimera was intermediate between 14-3-3\(\gamma\)/\(\xi\) and 14-3-3\(\gamma\)/\(\eta\) in terms of KSR1 binding, further indicating that sequence similarity at the CTS also correlates with isoform specificity. According to Obsil and co-workers (42), the flexible CTS folds back into the ligand binding pocket and can be displaced only by specific phosphopeptide sequences. Our results are compatible with this model, although, rather than a strictly autoinhibitory role, they suggest a positive role for the CTS in ligand discrimination by stabilizing specific interactions engaged by the core of the 14-3-3 protein. They are also in accordance with recent data from optimal docking area studies suggesting that, secondary to the phosphopeptide binding, general protein-protein interactions at the so-called “desolvation patches” and subsequent chain/loop rearrangements allow for isoform-specific ligand binding (36). In sum, our results suggest that contacts with the core of each 14-3-3 isoform mainly determine substrate specificity. Once the primary interaction has occurred, the isoform-specific CTSs seems to recognize bona fide ligands and stabilize the complex.
dimers, which indicates that the binding of KSR1 to 14-3-3-γ and, to a lesser extent, 14-3-3-β observed in our in vitro assays is direct and specific. Further, the KSR1-bound pool of 14-3-3-γ molecules did not dimerize (at least to a detectable level) with endogenous 14-3-3-ε, although, in agreement with previous data (36, 46), 14-3-3-γ formed dimers preferentially with that isoform (Figs. 6A and 7B). The role played by different 14-3-3 dimers in the regulation of KSR1 function awaits further investigation, but it could be relevant in coupling to other 14-3-3-binding proteins, altering its subcellular localization, and/or inducing conformational changes. Nevertheless, our data stress the notion of isoform specificity in ligand discrimination and also highlight the complex mechanisms that 14-3-3 proteins may use to achieve functional specificity.

Several reports have demonstrated that dimerization-deficient 14-3-3 mutants can bind their targets as strongly as wild-type 14-3-3 (47–50), and we have also shown here that KSR1 can bind a phospho-mimic, dimer-deficient 14-3-3-S59D mutant. Although Ser-297 phosphorylation is constitutive and does not change after Ras activation, the dominant site Ser-392 might be specific for pSer-392 (19), the remaining pSer-297 might not change after Ras activation, the dominant site Ser-392 gets dephosphorylated by PP2A, resulting in dissociation of the KSR1 bound monomeric 14-3-3 mutants can bind their targets as strongly as wild-type 14-3-3 mutants (36, 46), 14-3-3 bound monomeric 14-3-3 mutants (36, 46), 14-3-3 bound monomeric 14-3-3 mutants can bind their targets as strongly as wild-type 14-3-3 mutants (36, 46), 14-3-3 bound monomeric 14-3-3 mutants can bind their targets as strongly as wild-type 14-3-3 mutants (36, 46), 14-3-3 bound monomeric 14-3-3 mutants can bind their targets as strongly as wild-type 14-3-3 mutants (36, 46), 14-3-3 bound monomeric 14-3-3 mutants can bind their targets as strongly as wild-type 14-3-3 mutants (36, 46), 14-3-3 bound monomeric 14-3-3 mutants can bind their targets as strongly as wild-type 14-3-3 mutants (36, 46), 14-3-3 bound monomeric 14-3-3 mutants can bind their targets as strongly as wild-type 14-3-3 mutants (36, 46)

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