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RESEARCH ARTICLE

VRK2 anchors KSR1-MEK1 to endoplasmic reticulum forming a macromolecular complex that compartmentalizes MAPK signaling

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Abstract The spatial and temporal regulation of intracellular signaling is determined by the spatial and temporal organization of complexes assembled on scaffold proteins, which can be modulated by their interactions with additional proteins as well as subcellular localization. The scaffold KSR1 protein interacts with MAPK forming a complex that conveys a differential signaling in response to growth factors. The aim of this work is to determine the unknown mechanism by which VRK2A downregulates MAPK signaling. We have characterized the multiprotein complex formed by KSR1 and the Ser-Thr kinase VRK2A. VRK2A is a protein bound to the endoplasmic reticulum (ER) and retains a fraction of KSR1 complexes on the surface of this organelle. Both proteins, VRK2A and KSR1, directly interact by their respective C-terminal regions. In addition, MEK1 is also incorporated in the basal complex. MEK1 independently interacts with the CA5 region of KSR1 and with the N-terminus of VRK2A. Thus, VRK2A can form a high molecular size (600–1,000 kDa) stable complex with both MEK1 and KSR1. Knockdown of VRK2A resulted in disassembly of these high molecular size complexes. Overexpression of VRK2A increased the amount of KSR1 in the particulate fraction and prevented the incorporation of ERK1/2 into the complex after stimulation with EGF. Neither VRK2A nor KSR1 interact with the VHR, MKP1, MKP2, or MKP3 phosphatases. The KSR1 complex assembled and retained by VRK2A in the ER can have a modulatory effect on the signal mediated by MAPK, thus locally affecting the magnitude of its responses, and can explain differential responses depending on cell type.

Keywords VRK2 · KSR1 · MAPK · ERK · EGF · Cell signaling

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Abbreviations

ERK	Extracellular signal regulated kinase (MAPK3)
JIP	c-Jun interacting protein
JNK	c-Jun N-terminal Kinase
KSR1	Kinase suppressor of RAS 1
MAPK	Mitogen-activated protein kinase
MEK1	MAPK/ERK kinase 1 (MAP2K1)
VRK	Vaccinia-related kinase

Introduction

The specificity and variation in the cellular response to a common stimulation among cell types are probably determined by the composition of its downstream components in the signaling network [1], as well as protein interactions that can alter the balance among alternative response pathways. The spatial and temporal organization of cell signaling pathways require the formation of protein complexes that can determine the magnitude and subcellular localization of the complex and even the type of effect. These complexes are organized on modular scaffold proteins that interact with several components of signaling pathways. Scaffold proteins play a major role in intracellular signaling compartmentalization, and thus determine the temporal and local distribution of incoming signals [2, 3], as well as the fate of the response [4]. The organization of these signaling complexes is determined by their composition of interaction modules [5, 6]. Also, the assembly of complexes on scaffold proteins permits insulation of the pathway from noise signals that are originated in other signaling routes with which some components might be shared [7]. These complexes and their composition are starting to be identified as critical components of the so-called interactome [4] in the spatial and temporal regulation of cell signaling [1]. The organization of alternative complexes can be an important modulatory factor in growth factor responses, and might explain the differential responses observed among different cell types to a common stimulation.

Many intracellular signals are transmitted by different groups of kinases belonging to the MAPK families [8–11]. MAPK pathways are composed of three consecutive kinases, with the existence of several parallel pathways, each responding to a specific type of stimulation [10, 12]. But these kinases are also assembled on scaffold proteins that can determine the type and/or magnitude of the response [3, 7], as well as its spatiotemporal control [13] based on subcellular localization and interacting proteins, with either the scaffold or the kinases defining the pathway [5, 6]. The scaffold proteins organizing MAPK signaling are the best characterized, and among them are JIP1 [14], KSR1 [15, 16], paxillin, β-arrestin, or MORG1 [7]. Scaffold proteins allow the modular organization of signaling complexes in different subcellular compartments, therefore determining signal magnitude and specificity of the response [2, 6]. Not surprisingly, the study of new modulatory proteins of the MAPK pathway is an expanding research area [17, 18]. Their modularity allows a large functional flexibility that can determine the specificity of the biological effect depending on its context. In addition, these scaffold proteins interact with additional proteins that can modulate signals transmitted by the main pathway. Furthermore, the subcellular localization of these complexes might also be affected by interaction with proteins that are anchored on specific membranes, as is the case of VRK2 that can retain complexes bound to the endoplasmic reticulum (ER) [19–21], thus creating intracellular complexes with a restricted localization and/or function.

Kinases that appeared late in evolution such as the VRK family [22] are likely to play a modulatory role of signaling pathways that already exist in the cell and reflect the increase in the complexity in the organisms. Thus, late-appearing kinases are likely integrators of signaling networks already present in the cell and that appeared earlier in evolution. MAPK signaling is an early signaling pathway already present in yeast [2]. VRK2 is a member of the VRK family [23], which regulates p53 function [24, 25] and is associated with cell proliferation and cell cycle progression [26–29]. VRK2 has two isoforms: VRK2A (or VRK2) has 508 aminoacids and is anchored to ER membranes in the cytosol, while the second isoform VRK2B has 397 aminoacids and lacks the C-terminal region of isoform A; it is free and present in both nucleus and cytosol [30]. VRK2A, in addition to phosphorylation of p53 [30], is able to modulate the cellular response to hypoxia or growth factors [19–21]. VRK2A directly interacts with the JIP1 scaffold protein, retaining it in the ER fraction and forming a large signalosome that downregulates the stress response to hypoxia [19], or interleukin-1β mediated by JNK, and preventing the incorporation of c-Jun into the complex [20]. Also, VRK2A inhibits the signal from activated oncogenes such as erbB2, RAS^{G12V}, or $B\text{-}RAF^{V600E}$ mediated by ERK and assembled on the KSR1 scaffold protein, in which phosphorylation of ERK is blocked by the interaction of the complex with VRK2A [21]. In this work, we have studied the macromolecular organization of the VRK2A complex with KSR1 in order to characterize a subcellular signaling compartment and a modulator of mitogenic signaling. This large complex or signalosome contributes to the formation of intracellular signaling network and to signal compartmentalization.

Materials and methods

Plasmids

VRK2 was expressed from plasmids pCEFL-VRK2A, kinase-dead pCEFLVRK2A(K179E), and pCEFL-VRK2B [30]. Plasmids pCEFL-VRK2A (1–320), plasmids pCEFL-VRK2A (256–397), and plasmids pCEFL-VRK2A (364–508) have been previously described [19, 20]. Bacterial expression plasmids for GST-VRK2A and GST-VRK2B have been previously reported [30]. VRK1 was expressed from pCEFL-VRK1 [25]. Plasmid VHR was from

A. Alonso [31], while pEF-HA-VHR and plasmids expressing pRK5-HA-MKP1, pCMV-myc-HA-MKP2, and pRK5-HA-MKP3 were from R. Pulido (CIPF, Valencia, Spain). KSR1 was expressed from pCMV-Flag-KSR1 [32].

Cell lines, culture and transfections

The HEK-293T, HeLa, and MCF7 cells were grown in DMEM supplemented with 10 % fetal calf serum and antibiotics. Transfections were performed using the JetPEI reagent (Polyplus, Illkirch, France) or Lipofectamine 2000 (Invitrogen) as previously described [21, 30]. The total amount of DNA in transfections was kept constant by completion with the corresponding empty vector.

VRK2 and KSR1 knock-down by siRNA interference

The targeted sequence for VRK2 (GenBank NM_006296) was 5′-GCAAGGUUCUGGAU GAUAUUU-3′ (duplex siVRK2-06) (Dharmacon). Human Ksr-1 siRNA containing a pool of three target-specific 20- to 25-nt siRNAs was obtained from Santa Cruz (sc-35762). The siControl nontargeting siRNA pool (Dharmacon) was used as a negative control. Transfection of siRNA duplexes at a final concentration of 160 mM was carried out using Lipofectamine 2000 reagent (Invitrogen) [21]. Cells were processed for immunoblot 4 days after transfection. In assays of VRK2 depletion, cells were transfected with the reporter plasmid 72 h after siRNA transfection and EGF-stimulated 24 h later.

Antibodies

VRK2 was detected with a rabbit polyclonal antibody [19, 20, 30]. Calreticulin and KSR1 were detected with monoclonal antibodies from BD Biosciences. The HA epitope was detected with monoclonal HA11 from Covance (Emeryville, CA). FLAG epitope was detected with monoclonal M5 from Sigma (St. Louis, MO, USA). GST was detected with monoclonal B14 from Santa Cruz.

Membrane fractionation

HeLa cells were transfected with plasmid pCMV-Flag-KSR1 [32] and protein extracts prepared in lysis buffer: 250 mM sucrose, 20 mM HEPES pH 7.0, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA 1 mM EGTA, and protease inhibitors. The extract was passed through a 25-G needle and centrifuged at 720*g* to discard the nuclear fraction, and at 10,000*g* (Eppendorf 5415R centrifuge) to discard mitochondria. Aliquots of pellet and supernatant were kept for gel analysis. The supernatant was centrifuged at 100,000*g* (Beckman TLA100.3 rotor) for 1 h at 4 °C. The supernatant of these centrifugations was 300 µl. One-tenth of the supernatants was loaded in gels and indicated as soluble fraction (S), and the precipitate was completely loaded as microsomal fraction (P) for immunoblot analysis.

Immunoprecipitation, pulldown assays and immunoblots

Immunoprecipitations were made using 1.5 mg of total cell extracts and prepared in lysis buffer. To avoid non-specific interactions, cell extracts were preincubated with 30 µl of "GammaBind Plus Sepharose" beads (GE Healthcare) equilibrated in the same buffer as the extracts, and incubated for 1 h at 4 °C with orbital rotation. The beads were removed by centrifugation and the extract was incubated with the specific antibody indicated in the experiment for 3 h. Afterwards, 40 µl of GammaBind Plus Sepharose beads which have been previously blocked with seroalbumin (BSA) were added and incubated for 2–3 h or overnight at 4 °C with rotation. Next, the resin was washed several times in lysis buffer before processing for gel loading. The immunoprecipitate was fractionated by SDS–PAGE, and the gel transferred to a PDVF membrane, Immobilon-P (Millipore), for western blot analysis.

GST-tagged VRK2 proteins were pulled down by adding Glutathione Sepharose beads (GE Healthcare) to over-expressing cell lysates. Beads were washed five times with binding buffer and proteins were resolved in SDS–PAGE [21].

Detection of protein complexes by FPLC

In HeLa cells, endogenous VRK2 of KSR1 proteins were knocked down and their lysates used for isolation of protein complexes by high-pressure liquid chromatography. The whole-cell lysates were prepared in a buffer [containing 20 mM Tris–HCl pH 7.4, 137 mM NaCl, 2 mM EDTA, 25 mM β-glycerophosphate, 10 % (v/v) glycerol, and 1 % Triton-X100] with inhibitors of proteases and phosphatases (1 mM PMSF, 10 mg/ml aprotinin, 10 mg/ml leupeptin, 1 mM Na orthovanadate) as previously described [20]. The insoluble material was removed by centrifugation at 16,000*g* for 20 min. The soluble supernatant containing 1.5 mg protein was fractionated by FPLC in a Superose 12 10/300 GL column (GE Healthcare). HPLC was performed with an AKTA Prime model from Pharmacia-GE Healthcare equipped with a ChemStation software. The fractions were eluted running buffer (50 mM Tris–HCl, pH 7.5, 1 mM EDTA, 100 mM KCl) at a flow rate of 0.1 ml/min. Fractions of 0.2 ml were collected, precipitated and resolved on a 7.5 or 10 % polyacrylamide gel and immunoblotted. The column was calibrated with the following Molecular weight markers: bovine thyroglobulin (670,000), apoferritin from horse spleen (440,000), alcohol dehydrogenase from yeast (150,000), bovine serum albumin (66,000), and bovine carbonic

anhydrase (29,000), all from Sigma. The eluate was monitored at 280 nm.

Immunofluorescence and confocal microscopy

HEK-293T cells were grown on uncoated glass coverslips, placed in 60-mm plates. Cells were prepared as previously reported [30]. Fluorescence images were captured with a LEICA TCS SP5 DMI-6000B confocal microscope (Leica) using the following lasers: Argon (488 nm), DPSS (561 nm), and UV Diode (405 nm). Fluorescent images were captured with a ×63.0 lens zoomed in ×1.5–3 with a 1,024 × 1,024 frame and 600 Hz scanning speed. Scanner settings were maintained constant throughout all samples examined: pinhole (95.6 μm), lasers intensity and photomultipliers gain and offset. Images were analyzed with LEICA LAS AF (Leica) and imageJ (NIH, http://rsb.info.nih.gov/ij) software.

Results

VRK2 colocalizes with KSR1 in the endoplasmic reticulum

The subcellular localization of proteins can identify the compartmentalization and thus the potential signal distribution within cells. VRK2A colocalizes with the ER and VRK2B is free in the cytosol [30]. Therefore, it was determined by confocal microscopy whether the cytosolic KSR1 protein was also partly located in the ER or Golgi compartments. KSR1 by itself cannot be part of membrane fractions, because it lacks a transmembrane or hydrophobic region and its interaction has to be indirect through a membrane-bound protein. A fraction of the endogenous KSR1 was detected colocalizing with calnexin (Fig. 1a), an ER marker, but not with giantin (Fig. 1b), a Golgi marker. A similar result was observed in several human breast cancer cell lines (Fig. S1). Since VRK2 is partly located in the ER [30], it was tested to see whether it also colocalized with KSR1. KSR1 and VRK2 were detected partially colocalizing in the ER (Fig. 1c). These results indicated that there are two KSR1 subpopulations within the cell, one in the cytosol and the other colocalizing with calnexin and VRK2, which might be forming an intracellular complex bound to ER membranes.

To determine that a fraction of MEK1 can also be located in the ER, its colocalization with calnexin was determined. HeLa cells were transfected with HA-MEK1 and the overlap of its signal with calnexin is shown in Fig. 1d.

Next, cell extracts were fractionated in cytosolic and membrane-bound fractions and a significant amount of transfected Flag-KSR1 was detected in membranes where calreticulin, an ER protein, was also present (Fig. 1e), consistent with their colocalization by confocal microscopy. The amount of KSR1 in the membrane fraction represents approximately 10 % of the total KSR1 protein in the cell. In this particulate fraction, MEK1 and VRK2 were also detected (Fig. 1e), which indicated that the three proteins might be forming part of a single complex.

The C-terminus of VRK2 directly interacts with the CA5 region of KSR1

To characterize the interaction between VRK2A and KSR1, a pulldown experiment using different constructs spanning the VRK2A sequence was performed. KSR1 interacted with the VRK2A region comprised between residues 364–508, which is its C-terminal region (Fig. 2a). There are two active VRK proteins (VRK1 and VRK2) [23] with VRK2 having two alternative isoforms [30]. Therefore, the interaction between KSR1 and these VRK was determined in pulldown assays. KSR1 interacted exclusively with the full-length VRK2A isoform, but not with its shorter VRK2B isoform, and thus lacks the C-terminal region (residues 394–508) or the closely related VRK1 (Fig. 2b). Next, the direct interaction between VRK2A and KSR1 was characterized in pulldown experiments using GST-VRK2A and a series of ΔN- or ΔC-terminal KSR1 deletion mutants (ΔN-KSR1 and ΔC-KSR1, respectively) comprising different CA domains. Full-length VRK2A interacted strongly with the 402–873 region of KSR1, suggesting that the C-terminal half of KSR1, which includes the large CA5 pseudokinase domain [15], is mainly involved in the interaction. A weaker binding was also observed between the N-terminal half (residues 1–521) of KSR1 and VRK2A (Fig. 2c). Interestingly, both the K402-873 and K1-521 mutants include the CA4 domain, suggesting that residues in that region also contribute to the formation of the VRK2A/KSR1 complex. To further characterize the interaction, we next performed pulldown assays using the C-terminal region of VRK2A (residues 364–508) as bait. The VRK2A C-terminal region strongly interacted with the CA5 domain of KSR1 (K542-873 mutant), while a very weak association was observed with the N-terminal half of KSR1 (Fig. 2c). Collectively, these results indicate that VRK2A and KSR1 associate through interaction in their C-terminal halves. They also indicate that additional and weaker interactions through the N-terminal half of KSR1 can contribute to the stabilization of the complex.

The CA5 domain is also implicated in binding to MEK1/2 and, therefore, we next wanted to investigate whether MEK binding to KSR1 was required for the formation of the VRK2/KSR1 complex. To further confirm this hypothesis, we performed an in vitro dose-dependent binding assay using purified recombinant GST-VRK2A and His-CA5 (KSR1 residues 520–873) proteins. As the

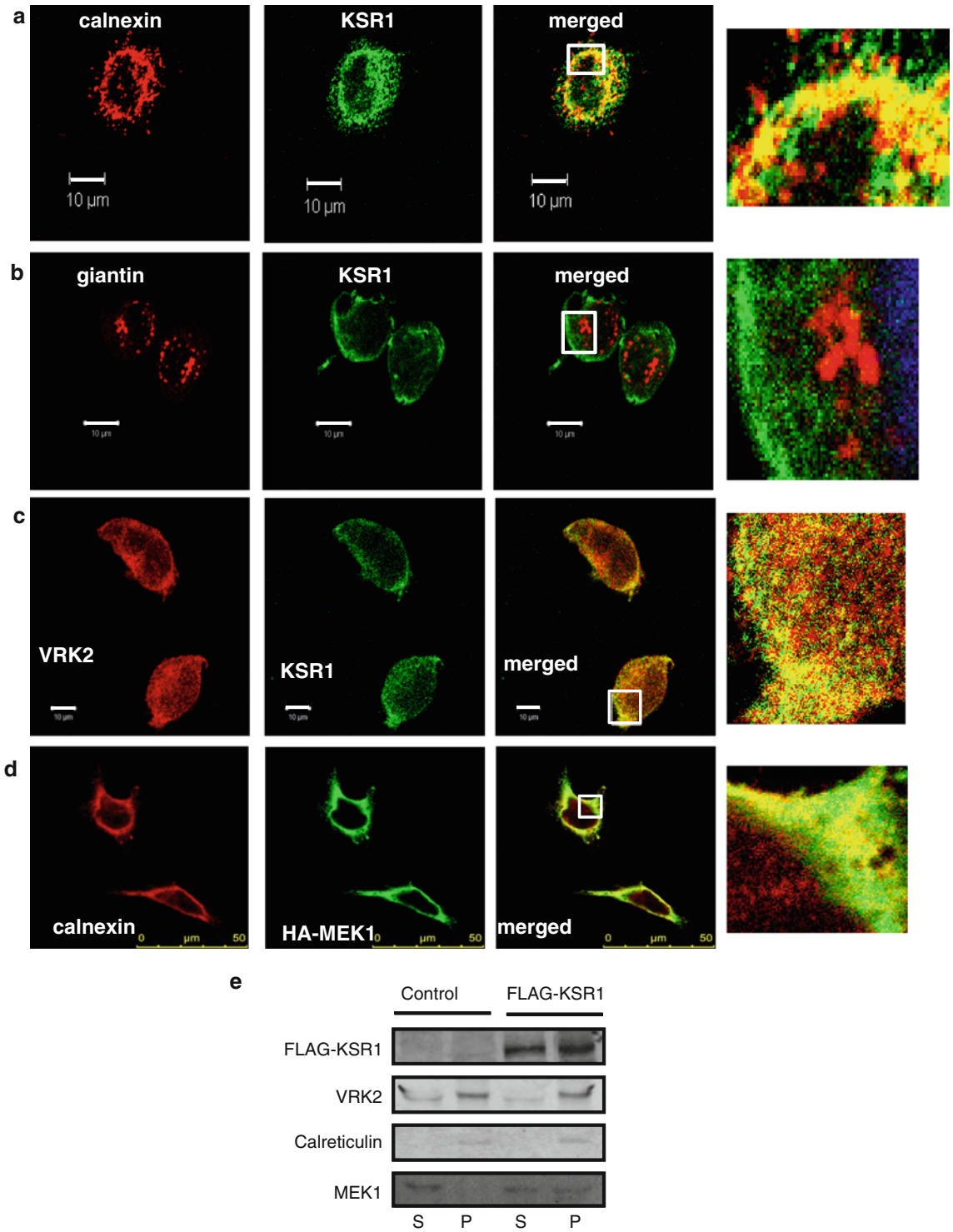

Fig. 1 Subcellular localization of VRK2 and KSR1 in membrane compartments in HeLa cells. **a** Colocalization of KSR1 (*green*) and calnexin (*red*), an endoplasmic reticulum marker. **b** Lack of colocalization of Giantin (*red*), a Golgi marker and KSR1 (*green*). **c** Colocalization of KSR1 (*green*) and VRK2 (*red*). VRK2 is located in endoplasmic reticulum [30]. *Bar* 10 μm. **d** Colocalization of calnexin with MEK1. HeLa cells were transfected with plasmid HA-MEK1 and its colocalization with endogenous calnexin determined by confocal microscopy. **e** HeLa cells were transfected with plasmid control or Flag-KSR1. Extracts were fractionated to separate membrane bound proteins from the soluble S100 fraction containing cytosolic proteins soluble fraction. In gels, one-tenth of the total soluble fraction and the total particulate fraction were loaded. The gel was used for immunoblot detection of VRK2, KSR1, MEK1, and calreticulin (ER marker) with the antibodies indicated in "Materials and methods"

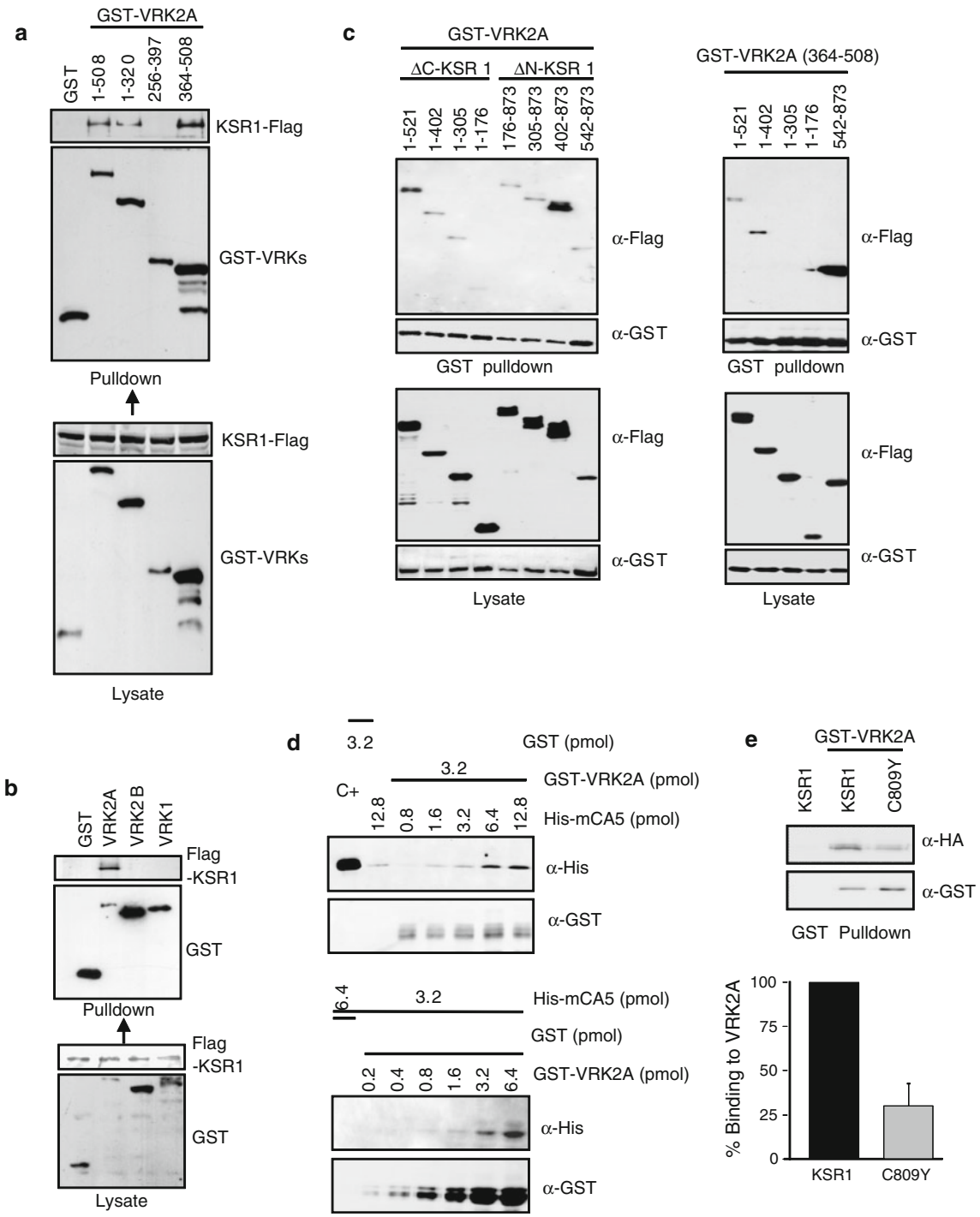

Fig. 2 Interaction of members of the VRK family with KSR1 and mapping of the interaction between VRK2A and KSR1. **a** Interaction of different fragments spanning VRK2A with KSR1. **b** Interaction of different VRK proteins (VRK2A, VRK2B, and VRK1) with KSR1. The interaction is specific for the VRK2A isoform. **c** Mapping the region of KSR1 that interacts with VRK2A. A GST-VRK2A constructs was used in a pulldown assay of deletion constructs from KSR1 N- and C-terminal regions. Interaction of VRK2A (*left*) and its C- terminal region (*right*) with different constructs of KSR1 in pulldown assay. **d** Interaction between VRK2A and the CA5 region of KSR1. Two types of pulldown assays were performed. In one, the amount VRK2A was fixed and assayed against increasing amount of KSR1-CA5 mutant (*top*). In the other, a fixed amount of KSR1-CA5 mutant was used against increasing amounts of VRK2A (*bottom*). **e** Binding of a C809Y KSR1 mutant to VRK2A. A GST-pulldown assay was performed using cell lysates overexpressing GST-VRK2A and HA-tagged KSR1 or KSR1-C809Y a mutant defective in MEK1/2 binding, and the amount of KSR1 protein bound to VRK2 quantified (*bottom graph*)

amount of His-CA5 was increased in the assay, so did the interaction in the pulldown binding assay (Fig. 2d, top), as increasing amounts of GST-VRK2A bound an increasing amount of His-CA5 domain (Fig. 2d, bottom). Taken together, these results indicate that MEK1/2 is not required for VRK2A to bind the CA-5 terminal region of KSR1. The structure of the CA5 region is altered by the C809Y mutation that alters the structure of its C-terminal region and interferes with its binding to KSR1 [33]. This KSR1-C809Y mutant still bound some VRK2A, although there was a reduction in the interaction (Fig. 2e). The lack of competition between VRK2A and MEK1 for interacting with KSR1 indicates that the three can form a complex.

MEK1 independently interacts with both KSR1 and VRK2

Besides being a KSR1 partner, MEK1/2 is known to interact with VRK2A [21]. Therefore, the direct interaction between KSR1 and MEK1 was characterized . To that end, we first determined the amount of endogenous MEK1/2 bound to different immunoprecipitated KSR1 deletion mutants. As expected, MEK1/2 was found associated with all ΔN KSR1 mutants while all ΔC KSR1 immunoprecipitates were devoid of any MEK1/2 (Fig. 3a), confirming that an intact CA5 domain is required for their interaction and that the pattern of binding is different from that detected with VRK2A. This CA5 region is the same large KSR1 domain that also interacted with VRK2A (Fig. 2b).

Next, it was determined whether MEK1 and VRK2 could interact directly in the absence of KSR1 using pull-down assays. Both isoforms of VRK2, VRK2A and VRK2B, interacted with MEK1 (Fig. 3b), suggesting that this interaction occurs by their common first 396 amino-acids. Next, the interaction of three regions of VRK2A with MEK1 was determined. The VRK2 N-terminal region between residues 1–320 interacted with MEK1 (Fig. 3c), and this region comprises the kinase domain of VRK2A.

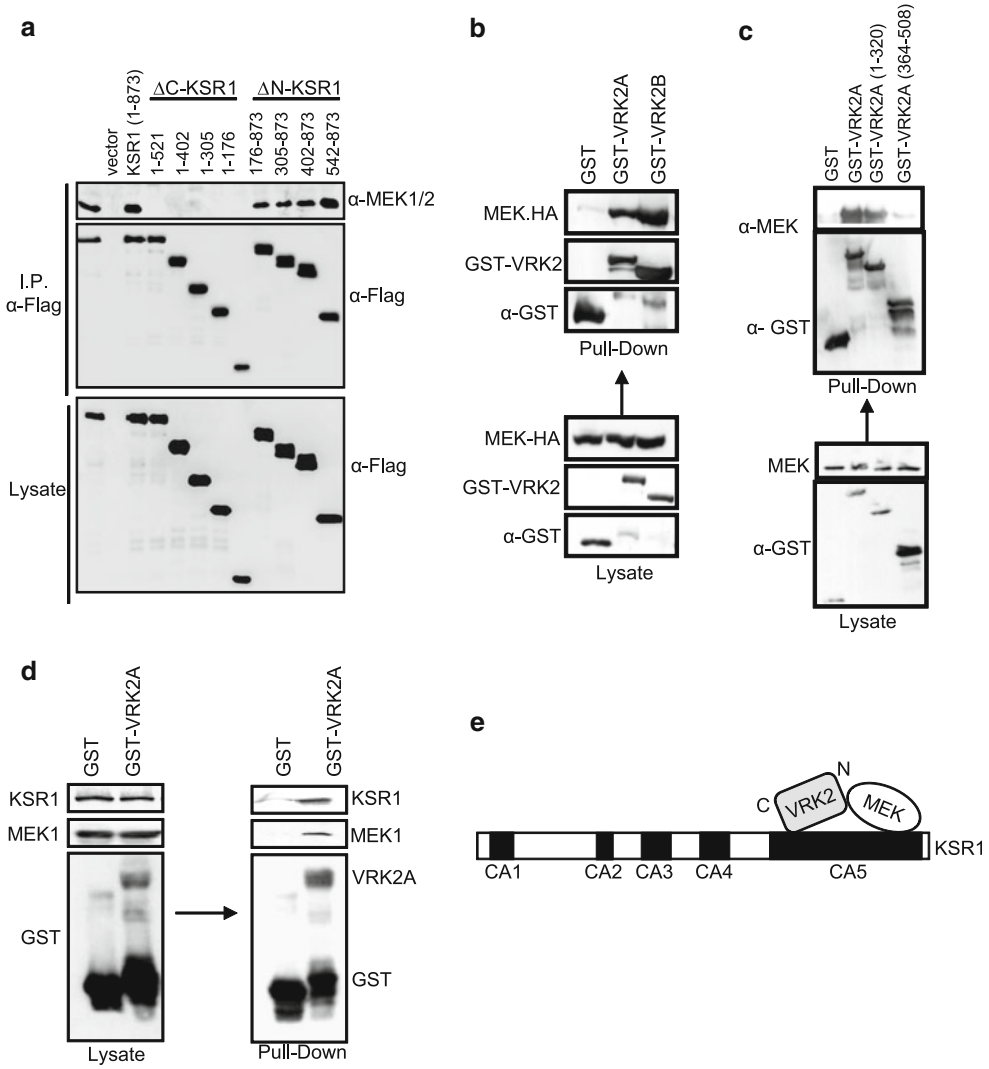

Fig. 3 **a** Interaction between MEK1/2 and KSR1 or VRK2A. A. HEK293T cells were transfected with deletion constructs of KSR1 which was immunoprecipitated with an anti-Flag antibody and the presence of endogenous MEK1/2 was determined in the immunoprecipitate (*top*). **b** Interaction of transfected HA-MEK1 with either VRK2A (1-508) or VRK2B (1-396). **c** Interaction of transfected VRK2 constructs with endogenous MEK1/2 identifying the N-terminal region of VRK2 as the interacting region. **d** Pulldown of proteins bound to VRK2A in HEK293T cells. Cells were transfected with a mixture of HA-MEK1, Flag-KSR1, and either GST or GST-VRK2A plasmids. The cell extracts were used for a pulldown and the proteins present detected with specific antibodies. **e** The complex formed by VRK2–KSR1–MEK1/2

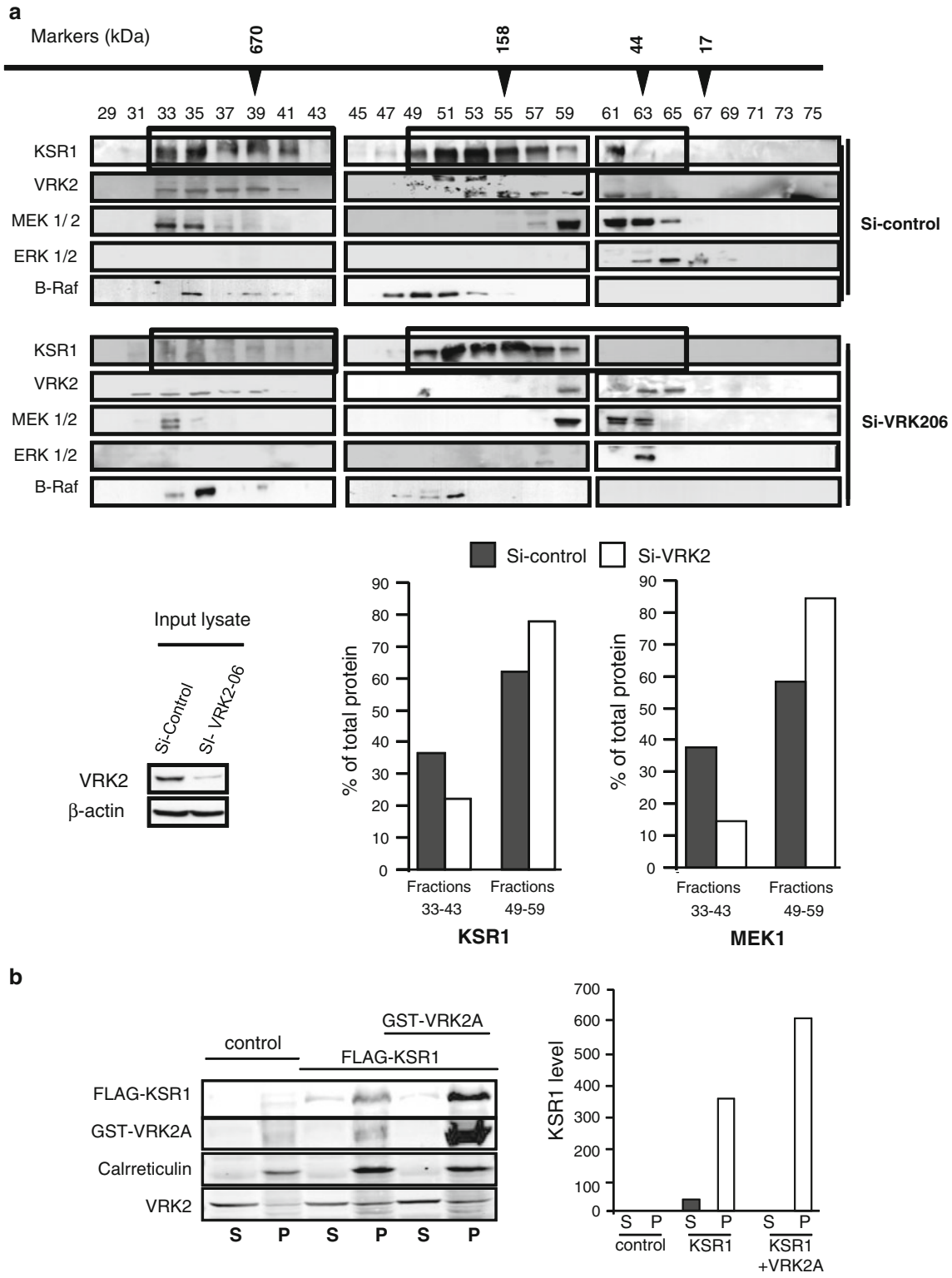

Fig. 4 **a** Detection of large intracellular complexes containing VRK2, KSR1, MEK11/2, Erk1/2, and RAF in Hela cells fractionated by FPLC. Control input extract is at the *bottom left*. At the *bottom right* is shown the quantification of KSR1 (*left*) and MEK1 (*right*) proteins in high molecular size (fractions 33–43) and mid–low size (fractions 49–59). **b** Overexpression of VRK2 increases the localization of KSR1 to the particulate fraction. HEK293T cells were transfected with combinations of Flag-KSR1 and GST-VRK2A plasmids. The transfected cells were lysed and fractionated by ultracentrifugation as indicated in "Materials and methods". The presence of each protein in the soluble and particulate fractions was determined. The amount of KSR1 in each fraction was quantified (*right graph*)

The detection of the three proteins in the complex was confirmed in a GST-VRK2A pulldown, in which both MEK1 and KSR1 were simultaneously detected (Fig. 3d), which is consistent with the lack of competition for binding to KSR1. This result indicated that VRK2A could potentially interact and simultaneously form a complex with both MEK1 and KSR1 in different regions in the molecule. A diagram of the signalosome complex formed by these three proteins is shown in Fig. 3e.

VRK2 and KSR1 can form intracellular complexes of high molecular weight

VRK2A has been shown to form large macromolecular complexes with scaffold proteins such as JIP1 [19, 20]. Therefore, it was tested whether KSR1 could also be included in large complexes, as was suggested by the subpopulation that colocalizes with KSR1 in HeLa cells (Fig. 1). For this aim, cellular extracts were fractionated by HPLC and the presence of proteins in different fractions was determined. In control cells, there were two identifiable KSR1 fractions, one in the 500- to 1,000-kDa (fraction H) and the other in the 100- to 300-kDa range (fraction M) (Fig. 4, top panel). Fraction H also contains MEK1. The knockdown of VRK2 resulted in the loss of KSR1 in fraction H but was without effect on fraction M (Fig. 4a, lower panel). These data suggested that VRK2 is likely to be necessary for the incorporation of KSR1 into large molecular weight complexes.

Also in these large complexes, the presence of MEK1-was detected, and its relative amount in large molecular size fractions compared with low molecular size was affected by siVRK2 (Fig. 4a), consistent with the interaction of both proteins. MEK1 is present in high molecular complexes with VRK2 and KSR1 and might represent a subpopulation of preassembled complexes, which is lost if VRK2A was knocked down. The functionality of these preassembled complexes would depend on their activation by the upstream kinase BRAF1 and subsequent association with its downstream effector ERK1/2. ERK1/2 was always detected in smaller fractions (L) corresponding to its free molecular weight, indicating that its interaction with the complex is not very stable or transitory. Thus, their participation in the complex is likely to be transient for activation and signal transmission.

Next, an inverse experiment was performed to determine whether overexpression of VRK2A will increase the amount of KSR1 in the membrane fraction. In this experiment, the soluble and particulate fraction were separated by ultracentrifugation at 100,000*g* (Fig. 4b). Overexpression of VRK2 increased the amount of KSR1 in the particulate fraction containing calreticulin by 100 % (Fig. 4b).

VRK2 prevents the incorporation of ERK to the KSR1 signaling complex

It is known that high levels of VRK2A downregulate the activation of ERK by MAPK on complexes assembled on the KSR1 scaffold protein [21]. Therefore, we tested whether this lack of ERK activation is a consequence of preventing the incorporation of ERK to KSR1 by the presence of VRK2A after activation of the MAPK pathway with EGF. For this aim, HEK293T cells transfected with Flag-KSR1 were treated with EGF, and the effect of VRK2 determined in the absence or presence of VRK2A. In cells in which VRK2A reached high levels by its overexpression, there was no incorporation of ERK to KSR1 complex as detected by immunoprecipitation (Fig. 5, right lane), but KSR1 and ERK were coimmunoprecipitated after EGF stimulation in the absence of high levels of VRK2A (Fig. 5, central lane).

VHR and MKP phosphatases do not interact with VRK2A

Assembled signaling complexes must be downregulated once the signal has been transmitted. The mechanism usually implicates several consecutive dephosphorylation

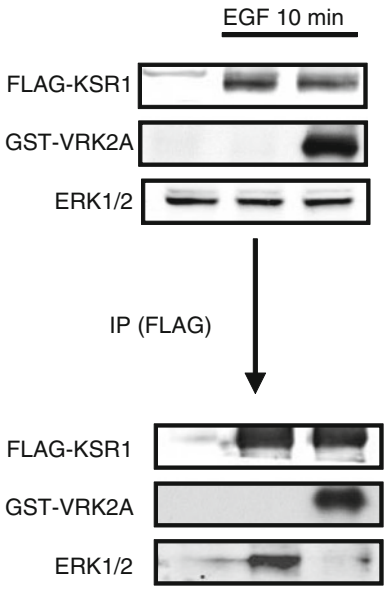

Fig. 5 Effect of high level of VRK2A on the KSR1-ERK1 interaction induced by EGF. HEK293T cells were transfected with plasmids pFLAG-KSR1 and pCEFL-GST-VRK2A as indicated, and transfected cells were stimulated with 10 ng/ml EGF in order to activate the MAPK pathway via the KSR1–MAPK complex. The formation of the KSR1-endogenous ERK1/2 complex was determined in the KSR1 immunoprecipitate in the absence (*center lane*) or presence of VRK2 (*right lane*)

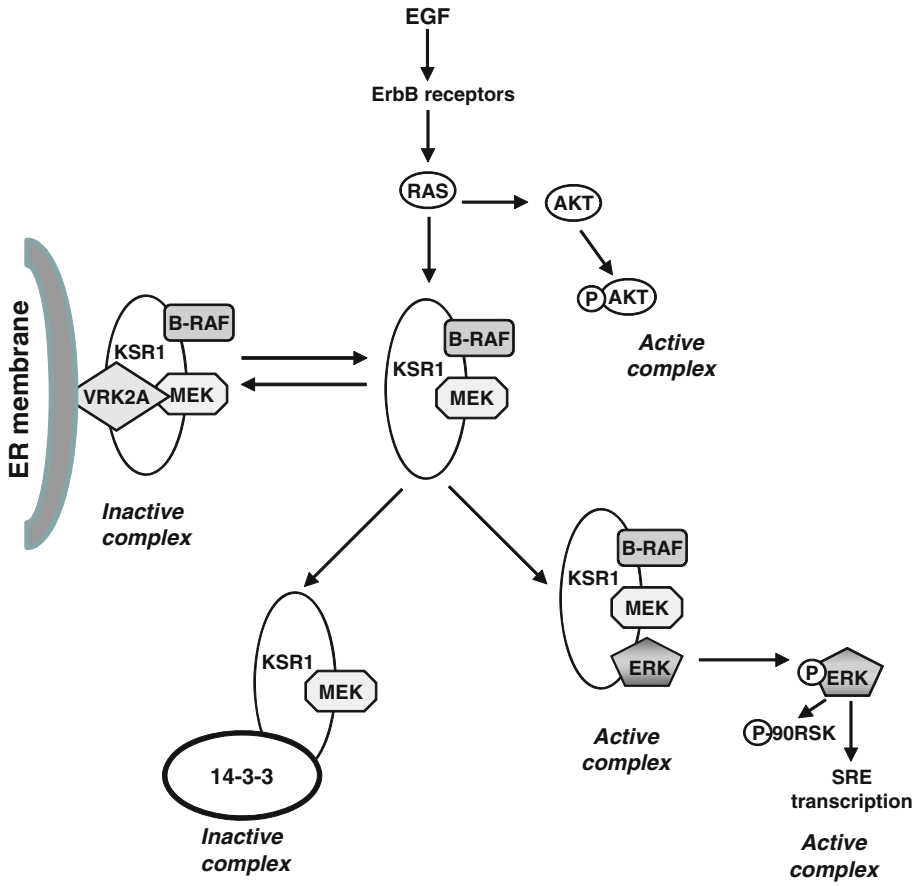

Fig. 6 Different types of alternative protein complexes or signalosomes formed by KSR1 implicated in the assembly and activation of the MAPK pathway containing MEK1. The active or inactive role of different signalosomes with respect to their composition is indicated

reactions through the intervention of one or more protein phosphatases. Among potential candidates is VHR, a dual phosphatase implicated in downregulation of RAS signaling [34, 35]. Another member of the VRK family, VRK3 has no kinase activity and might function as a scaffold protein [23, 36]. VRK3 has been shown to interact with the VHR phosphatase in the nucleus and in that way downregulates ERK signaling in the nucleus [36, 37]. Therefore, it was tested whether any of the VRK proteins could interact with VHR or some MKP phosphatase. Cytosolic VRK2A did not interact with VHR, MKP1, MKP2, or MKP3 phosphatases (Fig. S2).

VRK3 interacted strongly independent of the VHR construct and was used as positive control (Fig. S2a). VRK2A did not interact with VHR, but nuclear VRK1 and VRK2B interacted more weakly than the nuclear VHR–VRK3 interaction (Fig.S2a). Thus, we concluded that VHR interacts with nuclear members of the VRK family. The potential interaction between endogenous VRK2A and VHR was also ruled out in immunoprecipitation experiments (Fig. S2b). Endogenous VRK2A, the only VRK2 isoform expressed in 293T cells, did not interact with any of the phosphatases tested MKP1 (Fig. S2c), MKP3 (Fig. S2c), or MKP2 (Fig. S2d).

Discussion

Molecules that participate in intracellular signaling are likely to be organized at least partially into preassembled complexes needing only the specific input signal to initiate specific responses. If this is not the case, the diffusion kinetics, concentrations, and affinities of protein interactions will significantly affect the dynamics of signal responses, which can be critical when a fast response is required. The organization of multiprotein preassembled complexes is a mechanism for signal compartmentalization, as well as temporal regulation depending on the kinetics of assembly and disassembly of the components and regulators of the complex. Probably the best-known scaffold protein is JIP1 affecting the JNK-mediated responses [38]. But KSR1 is also a prototype organizer of signaling complexes that has proven more difficult to study. A role for signal compartmentalization based on KSR1 localization has been identified in neural tissues [39]. Some published data suggest that the cysteine-rich CA3 domain of KSR1 might be involved in direct interaction with lipids at the plasma membrane [51]. In spite of that, KSR1 proteins do not have a distinguishable hydrophobic or membrane interaction domain and, thus, its

association to ER membranes has to be indirect, mediated by another protein such as VRK2A. VRK2A has a C-terminal hydrophobic transmembrane region, anchored to ER [30]. The role of VRK2A in these complexes explains the presence of both KSR1 and MEK1 in large complexes of kDa [40]. This complex is reduced if either VRK2 is knocked down or both KSR1 and MEK1 move to fractions corresponding to its individual components in size pointing to their contribution to the stability of these large signalosomes. But it also indicates that the subpopulation bound to VRK2A has a different subcellular localization. However, the roles of MAPK signals in the ER do not have a well-identified function, but it is a possibility that they might be an important component in ER stress responses [41], a process associated with different pathologies including neurodegeneration [42, 43]. The complex is activated by RAF that is always in the S [40] (this work), thus the signal transmission from activated oncogenes such a RAS or ERBB2 is channeled by the soluble subpopulation.

It is very likely that these complexes have additional proteins that will contribute to the specificity of local effects which have not yet been identified, although one of the components is likely to be 14-3-3 proteins [32]. Lately, the identification of additional and novel regulatory proteins of MAPK signaling and affecting ERK-mediated responses has been greatly increasing. Several of them play a mainly inhibitory role that attenuated the signal. This effect can be mediated by protein–protein interactions with any downstream component, and as a consequence also alter the balance among alternative signaling pathways. The activated oncogene B-RAF^{V600E}, which forms a complex with KSR1 [44], is inhibited by heterodimerization with c-RAF [45]. KSR1 homodimerization or heterodimerization between MEK1 and MEK2 can mediate ERK activation and inhibition, respectively [46]. Several proteins directly interact with KSR1 and inhibit activation of ERK-mediated responses; among these are Nm23-H1 that induces KSR1 degradation [47], and 14-3-3 gamma [32]. In the case of 14-3-3 gamma, the complex is retained in the cytosol, and the interaction with KSR1 occurs through its CA3 domain [32]; thus it is different from the KSR1 interaction with VRK2A, which occurs by the CA5 domain. Thus; both can simultaneously inhibit ERK-mediated signaling in cytosolic and particulate fractions. The organization and composition of such large molecular complexes or signalosomes have not in general been characterized, and probably several additional components remain to be identified as well as their regulation.

The molecular organization of these alternative signalosomes or signaling protein complexes reflects the complexity of signaling networks, their interactions, and regulation, and can be important for understanding the variability of the responses observed in different environments or cell types. KSR1 is critical for the proliferative and oncogenic potential of cells [48], but also for induction of senescence by KRAS [49]; thus, its modulation can be an important control of this potential. A model of different functional alternatives is shown in Fig. 6. This seems to be the case in breast carcinomas, in which high VRK2 levels appear to downregulate proliferative signals without affecting survival signals in breast cancer and affecting signaling by the activation of the RAS-RAF-MEK1-ERK pathway [21]. The magnitude of a specific signal can thus be determined by the relative contribution of the different complexes that can be formed in a given cell, and the interacting proteins that alter the balance among alternative complexes. Thus KSR1–MEK1 can form active and inactive complexes depending on its subcellular localization, and its balance can be affected by the level of VRK2 protein that shifts it towards a higher proportion of inactive complexes retained in the ER and thus downregulates mitogenic signaling, as has been shown to occur on the mitogenic signal started in the EGF receptor and mediated by RAS and RAF, in which high levels of VRK2 inhibit the activation of AP1 dependent promoters [21]. This inhibitory effect has been positively correlated with breast cancer types positive for the estrogen receptor, and is partially lost, relieving inhibition in breast cancers overexpressing ERB2 [21]. This role of VRK2A is unlikely to be responsive to kinase inhibitors [50], since it is dependent on an interaction and not on the kinase activity. In addition, VRK2 also interacts with the scaffold JIP1 modulating the transcriptional response mediate by c-Jun [19, 20]. Thus, depending of the levels of KIP1 and KSR1, they might compete for interaction with VRK2A and so alter the balance between the activation of alternative signaling pathways such as those mediated by either JNK or ERK, which can have important biological consequences Fig. 6.

However, the interaction or sequestration of KSR1 to large complexes on the ER membranes can have an alternative role. The role of scaffold proteins such as KSR1 in allowing MAPK signaling is very sensitive to the level of the scaffold protein [2] and its excess results in inhibition of the pathway by sequestration of signaling components, but perhaps this inhibitory role may be relieved by retaining KSR1 anchored to a different set of proteins in a different subcellular compartment.

In this report, we have shown that a basic signalosome formed by KSR1 and MEK1 can be partially located to the ER by a direct interaction with both proteins, and that this complex can affect the magnitude of the signaling responses mediated by MAPK.

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Conflict of interest The authors declare they have no competing interests.

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