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Kinase Suppressor of Ras Couples Ras to the ERK Cascade during T Cell Development

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Ras signaling is critical for many developmental processes and requires the precise coordination of interactions among multiple downstream components. One mechanism by which this regulation is achieved is through the use of scaffolding molecules that coordinate the assembly of multimolecular complexes. Recently, the scaffolding molecule kinase suppressor of Ras (KSR) was isolated in genetic screens as a modifier of Ras signaling, although its contribution to regulating Ras-mediated activation of its different downstream effectors is not well understood. We have analyzed the role of KSR in linking Ras to the ERK cascade during positive selection. Our results demonstrate that KSR overexpression interferes with T cell development, an effect that requires the direct interaction between KSR and MEK. This functional effect correlates with the ability of KSR to uncouple Ras from the ERK cascade when overexpressed. The Journal of Immunology, 2004, 173: 986–992.

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2 Abbreviations used in this paper: DP, double positive; CRM, cysteine-rich motif; dGuo, 2′deoxyguanosine; dMEK, dead MEK; KSR, kinase suppressor of Ras; MIG, murine stem cell virus-internal ribosomal entry site-GFP; rFTOC, reaggregate fetal thymic organ culture; SP, single positive.
organ cultures (rFTOC). Overexpression of KSR in rFTOCs blocks the DP to SP transition during positive selection. The magnitude of this effect is comparable to that of a catalytically inactive MEK (dead MEK (dMEK)) in the same system, and the overexpression of KSR, as well as that of dMEK, blocks ERK activation in a thymoma cell line. Furthermore, structure-function analysis of KSR in this system showed that these effects of KSR overexpression require an interaction between KSR and MEK, suggesting that during positive selection of T cells, KSR links Ras signaling to ERK activation.

Materials and Methods

Generation of KSR constructs

A 4.1-kb EcoRI murine DNA fragment from mKsr pBluescript (provided by M. Therrien, Clinical Research Institute, Montreal, Canada) was subcloned into the EcoRI sites of the murine stem cell virus-internal ribosomal entry site-GFP (Mig) vector (provided by L. Van Paridj, Massachusetts Institute of Technology, Boston, MA). The KSR S392A, 1397A/V401A, R589M, and cysteine-rich motif (CRM) mutants were generated by PCR using the following primers: S392A, 5'-CTG AGG AGG ACA GAG GTA GCA GCA GCA GCA GAG and 5'-CTC TGC TCT CTC CAG AAG AAG; 1397A/V401A, 5'-CTC TGC TCT CTC CAG AAG AAG and 5'-CTC TGC TCT CTC CAG AAG AAG; R589M, 5'-CTC TGC TCT CTC CAG AAG AAG and 5'-CTC TGC TCT CTC CAG AAG AAG. These primers were paired with T3/T7 primers, and the resultant 4.1-kb PCR products were digested with EcoRI and HindIII. The bands obtained are the result of reverse-transcribed RNA. The identities of these PCR reaction products were confirmed by sequencing.

Analysis of mRNA expression

RNA samples from spleen and thymus from 4- to 6-wk-old C57BL/6 mice were generated using the Trizol system (Invitrogen Life Technologies, Carlsbad, CA). An equivalent amount of RNA for each sample was subject to reverse transcription, and the resulting cDNA was analyzed for KSR and GADPH expression by standard PCR and gel electrophoresis protocols to reverse transcription, and the resulting cDNA was analyzed for KSR and GADPH expression by standard PCR and gel electrophoresis protocols. The expression of KSR was determined by standardizing the band intensity from each reaction to that of a GADPH control as measured by densitometry using the AlphaImager densitometry analysis program (Alpha Innotech, San Leandro, CA). As a negative control, a reaction lacking reverse transcriptase was included to demonstrate that the bands generated are the result of reverse-transcribed RNA. The identities of the reverse-transcribed DNA products were confirmed by sequencing following subcloning into the TOPO-TA cloning vector (Invitrogen Life Technologies).

Intracellular staining

The 16610 D9 cells (provided by S. Hedrick, University of California, San Diego, CA) were cultured in RPMI 1640/10% FBS and infected with retroviruses by centrifugation at 1400 rpm at room temperature for 1 h. Twenty-four hours postinfection, cells were stained with 6 nM PMA for 10 min and harvested according to the protocol in Chow et al. (33). Following fixation, cells were stained with either rabbit or mouse anti-phosphorylated ERK Abs (NEB, Beverly, MA). Following staining with the primary Abs, cells were stained with either Cy5-conjugated anti-rabbit or Cy5-conjugated anti-mouse Abs (Jackson ImmunoResearch Laboratories, West Grove, PA) for 15 min and washed with 1× PBS/4% FBS. Samples were then analyzed by flow cytometry using a FACSCalibur (BD Biosciences) and the FlowJo analysis program (TreeStar Software).

Results

Overexpression of KSR blocks positive selection

Activation of Ras and of the ERK MAPK cascade is required during positive selection in the thymus (2, 3, 35), although ERK is probably not the only Ras downstream effector implicated in this process (36). Therefore, the identification of molecules necessary for coordinating and facilitating the activity of this pathway is important to understanding Ras function. Because it has been shown that a number of scaffolding molecules play a role in regulating the activity of Ras in different developmental models, we decided to test whether one of these molecules, KSR, which is normally expressed in all thymic populations (data not shown), is involved in the regulation of Ras MAPK activity during positive selection.

rFTOC of retrovirally transduced precursors provides a kinetically sensitive way to test this hypothesis. The use of fetal thymocytes from embryonic stages E14.5 to E16.5 allows transduction of precursors during the proliferative phase post β-selection (double-negative to DP transition), so that the gene of interest is expressed in DP thymocytes (37). Furthermore, the use of donor thymocytes derived from nonselecting background matings, MHC+, minimizes the background of cells positively selected before the transgenes can be expressed in the rFTOC. The infected thymocytes are immediately reaggregated with stromal cells derived from deoxyguanosine-treated E15.5 fetal thymic lobes, and cultured in rFTOC for up to 12 days to follow the kinetics of thymocyte differentiation from the DP to the CD4 and CD8 SP stages.

To investigate the role of KSR during thymocyte development, these rFTOC cultures were analyzed for TCRβ and GFP expression (Fig. 1A), and the TCRβhigh populations were subsequently analyzed for CD4 and CD8 expression (Fig. 1B). Two to five individual lobes were analyzed per time point in each experiment, and the results of individual lobes for two representative experiments are shown in Fig. 1C, in which we plot for each lobe, the ratio of the total number of TCRβhigh cells to the number of...
TCRβlow-mid DP cells for both the GFP− and GFP+ populations. In each experiment in this system, the GFP+ cells serve as an additional internal control for the GFP+ or GFP− populations. KSR infection, unlike Mig, results in a dramatic reduction in the percentage of TCRβhigh cells relative to uninfected cells. B. Analysis of the CD4/CD8 profiles within the TCRβhigh populations reveals that KSR overexpression results in an increase in the percentage of immature, DP cells, and a reduction in the total percentage of mature, single-positive cells, without alterations in the CD4/CD8 ratios. C. The y-axis is labeled as # TCRβhigh/TCRβDP, which represents the ratio of the number of TCRβhigh cells to the number of DP cells within the TCRβlow-mid populations. This figure shows that KSR infection reduces the fraction of mature TCRβhigh cells relative to empty vector-infected or uninfected cells. Each GFP− and GFP+ pair represents an individual lobe with the results of two different experiments analyzed at day 9 shown. Both experiments were performed using MHC+ donor thymocytes. D. Ratio of TCRβhigh cells in GFP+ vs GFP− populations for lobes infected with Mig or KSR. In vector-infected lobes, the ratio is between 1 and 1.7, while for KSR-infected lobes, the ratio is significantly decreased. Each bar is the average of the different lobes in each experiment (between 2 and 5). E. Analysis of TCR and HSA expression in thymocytes after 6 days of rFTOC. Thymocytes were infected with empty Mig or Mig/KSR, reaggregated, and cultured for 6 days.

FIGURE 1. KSR overexpression affects thymocyte development. rFTOCs overexpressing KSR and the control vector Mig were analyzed for the expression of TCRβ, GFP, CD4, and CD8 following 9 days of culture. A. The numbers indicated above each quadrant represent the percentage of the TCRβhigh cells in either the GFP− or GFP+ populations. KSR infection, unlike Mig, results in a dramatic reduction in the percentage of TCRβhigh cells relative to uninfected cells. B. Analysis of the CD4/CD8 profiles within the TCRβhigh populations reveals that KSR overexpression results in an increase in the percentage of immature, DP cells, and a reduction in the total percentage of mature, single-positive cells, without alterations in the CD4/CD8 ratios. C. The y-axis is labeled as # TCRβhigh/TCRβDP, which represents the ratio of the number of TCRβhigh cells to the number of DP cells within the TCRβlow-mid populations. This figure shows that KSR infection reduces the fraction of mature TCRβhigh cells relative to empty vector-infected or uninfected cells. Each GFP− and GFP+ pair represents an individual lobe with the results of two different experiments analyzed at day 9 shown. Both experiments were performed using MHC+ donor thymocytes. D. Ratio of TCRβhigh cells in GFP+ vs GFP− populations for lobes infected with Mig or KSR. In vector-infected lobes, the ratio is between 1 and 1.7, while for KSR-infected lobes, the ratio is significantly decreased. Each bar is the average of the different lobes in each experiment (between 2 and 5). E. Analysis of TCR and HSA expression in thymocytes after 6 days of rFTOC. Thymocytes were infected with empty Mig or Mig/KSR, reaggregated, and cultured for 6 days.

FIGURE 2. Time course analysis of the effect of KSR on T cell development in rFTOCs. rFTOCs derived from E15.5 B6D2F1 fetal thymocytes overexpressing KSR and the control vector Mig were analyzed for the expression of TCRβ, GFP, CD4, and CD8 following 6, 8, and 12 days of culture. A. The numbers indicated above each quadrant represent the percentage of TCRβhigh cells in the GFP+ or GFP− populations. At all stages of development analyzed, KSR infection results in a reduction in the fraction of mature, TCRβhigh cells. B. The CD4/CD8 profiles of the TCRβhigh populations from A are shown, with the percentages of cells in each population indicated within each quadrant. KSR overexpression in rFTOCs does not alter the CD4/CD8 ratios, but an increase in the percentage of immature DP CD4+CD8− cells is observed at all time points.

skewing in the generation of CD4 vs CD8 TCRβhigh cells in this system (Fig. 1B). The blockade in positive selection seems to occur at early stages in the process, because both TCRβhigh HSAhigh and TCRβlow HSAlow populations are affected (Fig. 1E). To test whether this decrease was due to an actual block in positive selection, or just to a delay in the appearance of TCRβhigh cells in the KSR-infected thymocytes, we performed time course experiments. As shown in Fig. 2, the decrease in the generation of TCRβhigh cells was already seen at day 6 of culture and was maintained at later time points (day 12). This effect was also observed in similar experiments as early as day 3 of culture (data not shown).

As this phenotype is reminiscent of the block in positive selection obtained by the overexpression of dominant-negative forms of Ras and MEK in transgenic mice (2, 3, 35), we decided to directly compare the effect of overexpressing KSR and the dominant-negative (dMEK) used in the original studies (MEKΔK7A) (35). E15.5 fetal thymocytes from C57BL/6 and MHC−° mice were infected in parallel with dMEK, KSR, and Mig, reaggregated, and allowed to develop for 9 days. Two representative rFTOC experiments are shown in Fig. 3. As shown in Fig. 3A, infection with both dMEK and KSR results in at least a 2-fold reduction in the percentage of TCRβhigh cells. In addition, both dMEK and KSR overexpression result in a reduction in the percentage of SP cells and an increase in the percentage of DP cells (Fig. 3B). Fig. 3C depicts the graphical representation of all the analyzed lobes in these experiments and reveals that infection with dMEK or KSR results in the reduction of mature TCRβhigh cells. These results suggest that KSR functions in a manner similar to dMEK, blocking positive selection by inhibiting activation of the MAPK cascade.

To directly test whether KSR could affect activation of the MAPK cascade, the thymus-derived cell line 16610D9 (38) was infected with dMEK, KSR, and Mig. Twenty-four hours postinfection, cells were stimulated for 10 min with 6 nM PMA to activate ERK, and the levels of phosphorylated ERK were analyzed by intracellular staining (in Materials and Methods (33)). Stimulation with PMA under these conditions results in optimal ERK
TCR/H9252 at day 8 and analyzed for GFP and TCRfi Abs and by in vitro kinase assays (data not shown). Specific activation in T cells, as assessed by Western blot with phospho-TCR populations. Two different rFTOCs are shown, demonstrating that, unlike the control vector Mig, infection with dMEK or KSR reduces the fraction of TCRβstop, mature cells. B. The CD4/CD8 distribution of TCRβstop cells shows no significant alteration in the CD4/CD8 ratios and an accumulation of TCRβstop DP cells in the KSR and dMEK rFTOCs. C. The ratio of total numbers of TCRβstop vs TCRβlow-mid DP thymocytes demonstrates that dMEK and KSR infections decrease the generation of mature cells. Two different experiments are shown, and each pair of GFP− and GFP+ points represents an individual lobe. D. The overexpression of dMEK and KSR blocks the activation of ERK in a T cell line. The 16610 D9 thymoma cells were infected with retroviruses encoding the designated constructs. Twenty-four hours postinfection, cells are either left untreated (U) or stimulated (P) with 6 nM PMA for 10 min and analyzed by intracellular staining for ERK phosphorylation and activation. The y-axis represents the levels of phosphorylated ERK (α-pERK), and the x-axis designates the levels of GFP expression. Gates are drawn on each histogram such that the GFP+ population is on the right side of the vertical axis. Cells infected with KSR or dMEK show an inhibition in ERK activation in the GFP− population.

**Structure-function analyses of KSR domains**

The mechanism by which the overexpression of KSR blocks development and ERK activation most likely involves the formation of nonfunctional complexes with other proteins in the Ras/MAPK signaling cascade. To determine the nature of these complexes, a series of KSR structural mutants were generated (Fig. 4). In particular, our studies have focused on the membrane localization domain, elements important for the association of KSR with other molecules, and the kinase domain.

The CA3 domain is important for the membrane localization of KSR, and in some systems functions has the same effect as wild-type KSR (39, 40). The CRM mutant converts the cysteines at aa positions 359 and 362 (within the CRM/CA3 domain) to serines. This mutant blocks the ability of KSR overexpression to augment Ras signaling in *Xenopus* oocytes (25, 39). Sites important for the interaction of KSR with the molecules c-TAK, 14-3-3, and MEK were also mutated. KSR may require membrane localization to participate in growth factor receptor signaling (18), and this translocation is regulated by both c-TAK and 14-3-3 (28). Mutations of S392 and I397/V401 to alamines interfere with the c-TAK and 14-3-3 interactions, respectively, resulting in dominant-positive forms of KSR that constitutively translocate to the membrane (28). Another molecular interaction important for KSR activity is its constitutive association with MEK. A KSR loss of function mutation corresponding to the cysteine at aa position 809 was initially identified in *C. elegans* (16). Mutations at this site abrogate the ability of KSR to interact with MEK, and consequently, its ability to assist in Ras signaling (21).

Based on its homology to Raf kinase, KSR was initially proposed to function as a kinase (41). However, the murine form of KSR lacks the conserved lysine at position 589 that corresponds to the Mg2+-ATP-binding motif that is important for phosphotransfer (15). It has been proposed that mutations at this site block the kinase ability of KSR (41–43), but it has also been suggested that this site is important for mediating the interactions of KSR with other molecules, rather than kinase activity (18). To investigate the importance of this site during T cell development, the arginine at position 859 was mutated to methionine to generate the R859M mutant. A R859K mutant was also generated to determine whether the kinase function is important because this mutant contains the conserved lysine in the Mg2+-ATP binding region.

Once generated, all of the KSR mutants (see Fig. 4A) were subcloned into Mig and tested for their effects on ERK activation using 16610D9 cells, as described above. Fig. 4B shows that, with
the exception of C809Y/Mig and CA3/Mig, overexpression of each of the mutants reproduces the inhibition of PMA-induced ERK activation mediated by KSR in wild-16610D9 cells. Overexpression of all mutants was confirmed by Western blot (data not shown).

Once screened for their effects in the ERK activation assay, S392A/Mig, C809Y/Mig, CA3/Mig, and the CRM/Mig mutants were tested for their effects on T cell development. These mutants were chosen so that representatives of both types of mutants (those that mimic the effects of KSR as well as those that do not) were analyzed. The effects of the mutants were analyzed between days 7 and 11 of culture by examining TCR<sup>+</sup>/H<sup>9252</sup> levels in rFTOC assays, as described above. As seen previously, KSR overexpression results in a decrease in the percentage of TCR<sup>+</sup>/H<sup>9252</sup> cells (Fig. 4, C and E). Among the KSR mutants tested, CRM/Mig (data not shown) and S392A/Mig had comparable effects to wild-type KSR. In contrast, the overexpression of CA3/Mig (data not shown) and C809Y/Mig did not alter the development of mature T cells in the rFTOC (Fig. 4, C and E). The CD4/CD8 profiles within the uninfected and infected TCR<sup>+</sup>/H<sup>9252</sup> high populations were also examined, demonstrating that as seen with TCR<sup>+</sup> expression, the S392A/Mig and CRM/Mig (data not shown) mutants behave similarly to KSR, while only the CA3/Mig (data not shown) and C809Y/Mig mutants do not behave like KSR (Fig. 4D). This analysis suggests that of the KSR domains analyzed, only the MEK interaction domain is required to block T cell development in rFTOCs, while the membrane localization and the kinase domains are dispensable for this effect. Furthermore, the isolated expression of the membrane localization domain (CA3) does not affect this process. These results also demonstrate that the ability of the mutants to interfere with ERK activation correlates with the ability to block T cell development.

**Discussion**

Ras signaling is central in the regulation of cell growth, survival, and differentiation. Ras controls these processes by activating numerous downstream effector pathways, including the MAPK cascade (44, 45). Although this cascade is the best-defined downstream Ras effector pathway, abundant evidence shows that it is insufficient to recapitulate Ras function in numerous processes,
including mammalian cell transformation, T cell activation, and development (7, 36, 46). The mechanisms that regulate which Ras effectors are recruited are unclear, but the isolation by genetic screens of a number of proteins that function as scaffolds suggests that they may be integral for the effective activation of specific signaling cascades (47).

Scaffolds coordinate an appropriate response by interacting with other proteins and organizing them into a multimolecular complex. This association is important for fostering interactions with molecular partners as well as sequestering them from inhibitory molecules. To understand in more detail how Ras functions during positive selection of thymocytes, we have addressed the role of KSR in this process. KSR was identified as a modulator of Ras signaling in genetic screens performed in D. melanogaster, and is physiologically expressed in the thymus. Our results show that overexpression of KSR disrupts T cell development, and that this effect correlates with its ability to alter MAPK activation downstream of Ras.

In invertebrates, a number of putative scaffolding proteins capable of affecting Ras signaling have been identified, some of which do not generate obvious phenotypes when mutated. This may be due to their role as fine-tuning components of their respective pathways rather than as primary signaling molecules. In accordance with this observation, KSR mutations do not produce strong phenotypes in flies, and a knockout of KSR does not generate an obvious phenotype in developing murine thymocytes (48). However, upon closer examination, it is clear that KSR does affect signaling in thymocytes, because ERK signaling is compromised in mutant animals (48). The lack of a developmental phenotype in the KSR knockout transgenic animals is likely to be due to the expression of a second KSR gene in the thymus. This hypothesis is supported by the identification of a second KSR gene in C. elegans, both of which have partially redundant functions (49).

Therefore, to further understand the role of scaffolding molecules in thymocytes, approaches other than gene disruption may be informative. For example, in Drosophila, sensitized backgrounds in which the levels of the molecules of interest are below a critical threshold provide a means to study molecules with more subtle effects. As scaffolding molecules coordinate multimolecular complexes, another approach to investigating their function entails overexpression studies. For example, the overexpression of wild-type connector enhancer KSR in the Drosophila eye imaginal disc has a mild dominant-negative effect on Ras signaling, most likely due to the titration of connector enhancer KSR-interacting proteins that are necessary for Ras signal transduction (50).

To study the role of KSR in regulating Ras signaling during positive selection, we chose an ex vivo approach using rFTOC. This system reproduces in vivo fetal T cell development (51) and makes it possible to introduce genes in the developing T lymphocytes using retroviral-mediated gene transfer. This system is useful for studying the function of scaffolding molecules because these molecules can be significantly overexpressed as compared with endogenous protein levels. Furthermore, this method also allows for the manipulation of a large number of multiple constructs, a facet that is prohibitively time consuming by traditional transgenic approaches.

During fetal development, overexpression of KSR has an effect upon T cell development, resulting in a block in positive selection. This phenotype is similar to that observed in adult thymocytes following the overexpression of a dominant-negative form of Ras or of catalytically inactive MEK (dMEK) (2, 3), and to the effect of overexpressing the dMEK construct in rFTOCs. Despite the effect on the generation of single-positive thymocytes, we did not observe any alteration in the CD4/CD8 ratios, although this system is good at detecting such differences (37). Although it has been suggested that the intensity of MAPK signals can contribute to this lineage commitment decision, these results, as well as our previous experiments in transgenic mice, argue against this possibility (reviewed in Refs. 36, 52, and 53).

The developmental effects observed by overexpressing KSR correlate well with its biochemical effects: overexpression of KSR blocks ERK activation as effectively as overexpression of dMEK. Interestingly, there is a difference between these two constructs. Although the effect of dMEK seems to be linearly related to the amount of transgene expressed (which roughly corresponds with the amount of GFP expressed by the infected cells), KSR blocks the cascade in an all-or-none fashion. Cells with low expression seem to activate ERK normally, but after a certain threshold level, the cascade is blocked completely. This suggests that the effect of KSR is due to the titering away of a limiting component in the signaling cascade.

To investigate the structural elements of KSR important for this effect, a series of mutations within KSR were generated. Mutations of the membrane localization motif, the kinase domain, the c-TAK, or 14-3-3 interaction domains do not reverse the effects of KSR overexpression in our assays, suggesting that these domains are not critical for the overexpression phenotype. In addition, generating a predicted kinase active form of KSR has no effect in our assays. However, mutations predicted to disrupt the interaction between KSR and MEK render the construct incapable of blocking thymocyte development. Thus, in our system, the effect of KSR appears to be due to the sequestration of MEK. We propose that in thymocytes, the association between KSR and MEK occurs within the cytoplasm before KSR membrane translocation because membrane localization of KSR is not required for its inhibition of Ras signaling.

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