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

Micheline N. Laurent, Danny Maria Ramirez and José Alberola-Ila

*J Immunol* 2004; 173:986-992; doi: 10.4049/jimmunol.173.2.986

http://www.jimmunol.org/content/173/2/986

---

**References**  
This article cites 53 articles, 21 of which you can access for free at:  
http://www.jimmunol.org/content/173/2/986.full#ref-list-1

**Subscription**  
Information about subscribing to *The Journal of Immunology* is online at:  
http://jimmunol.org/subscription

**Permissions**  
Submit copyright permission requests at:  
http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**  
Receive free email-alerts when new articles cite this article. Sign up at:  
http://jimmunol.org/alerts
Kinase Suppressor of Ras Couples Ras to the ERK Cascade during T Cell Development

Micheline N. Laurent, Danny Maria Ramirez, and José Alberola-Ila

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.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Division of Biology, California Institute of Technology, Pasadena, CA 91125

Received for publication November 13, 2003. Accepted for publication May 12, 2004.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 Address correspondence and reprint requests to Dr. José Alberola-Ila, MC147-75, California Institute of Technology, 1200 E. California Boulevard, Pasadena, CA 91125. E-mail address: alberola@caltech.edu

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.

Copyright © 2004 by The American Association of Immunologists, Inc.
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. Therien, 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 Paris, Massachusetts Institute of Technology, Boston, MA). The KSR S392A, 1397A/V401A, R589M mutant forms and cysteine-rich motif (CRM) mutant forms were generated by PCR using the following primers: S392A, 5′-CTT CGG AGG ACA GAG GCA GTC CGC TCG TAT ATC and 5′-GAT ATC TGA CGG TAC TGC CTC TGT CCT CCC AAG; 1397A/V401A, 5′-CCG TCA GAT GCC AAC AAC CCA GGC AGC GAC GCA GCA GAG and 5′-CTC TGC TGC TCT GCT GCC TGG TGG GTT GGC ATC TGA CGG TGG TGC GGT GGC ATT ATG CTG CAG GAG ACC GCAC GAT CTC GCC-3′; R589M, 5′-GGA GAG GTG GGC ATT ATG CTG CAG GAT AGC-3′; and 5′-GTC CAT CTC CAG CAG AAT GAT CAC CAC CTC GCC-3′; 1397A/V401A, 5′-GTC CAT CTC CAG CAG AAT GAT CAC CAC CTC GCC-3′; C809Y, 5′-GTC GAG GAC ATC CTG TCT GCC TAC TGG GCT TTC CAG CAG and 5′-GTC CTG CAG AAT GAT CAC CAC CTC GCC-3′; and CRM, 5′-GTC AAG TCC AAA CAC TCC AGG TTA AAA TGC C and 5′-GCT GGA GTG TIT GTA GGA CTT AAA GCC C DNA sequences encoding the mutated codons are underlined in boldface. These primers were paired with T3/T7 primer and reverse primers to generate the 4.1-kb fragments (as listed in the text and figure legend). Thymic lobes were digested with 2 mg/ml T4 collagenase in RPMI 1640, 1× PBS, and 4 mM deoxyguanosine (dGuo; Sigma-Aldrich, St. Louis, MO) at 37°C/7% CO2 for 1 h (34). The lobes were then washed in excess medium supplemented with 1.35 mM 2′deoxyguanosine (dGuo; Sigma-Aldrich, St. Louis, MO) at 37°C/7% CO2 for 1 h (34). The lobes are placed onto MilliPore (Bedford, MA) 050 PICM filters within a six-well dish (1 ml of 1.35 dGuo/well) and incubated at 37°C/7% CO2 for 5 days. After dGuo treatment, lobes are washed three times in excess DMEM/10% FBS, once with 1× PBS, followed by 0.05% trypsin/EDTA treatment for 40 min to dissociate stromal cells from the thymic matrix. Postdissection, cells are incubated in DMEM/10% FBS medium at 37°C/7% CO2 until donor thymocytes are prepared (described below).

On the day of reaggregation, donor thymocytes are microdissected from E15.5−E16.5 fetuses (from C57BL/6, B6D2F1, or MHC−/− pregnant females) and stained with type IV collagenase (Sigma-Aldrich; 2 mg/ml) prepared in RPMI 1640/20 mM HEPES at 37°C/7% CO2 for 0.5 h. Two million thymocytes are cultured with retroviral particles in a 24-well dish and centrifuged (1400 rpm) at room temperature for 1 h. The retroviral supernatant was replaced with DMEM/10% FBS, and cells were cultured for a minimum of 2 h at 37°C/7% CO2. To reaggregate lobes, 2 × 105 donor thymocytes are combined with 2 × 105 host stromal cells in a total volume of 0.7 μl of DMEM/10% FBS and placed on filters for air-liquid interphase culture and incubated for the designated time periods.

Analysis of thymocytes from rFTOC

Lobes were harvested and treated with 2 mg/ml T4 collagenase in RPMI 1640/10 mM HEPES at 37°C/5% CO2 for 1 h in a 96-well dish. Cells were centrifuged and resuspended in 1× HBSS/4% FBS plus dilutions offluorophore-conjugated Abs to TCRβ, CD4, and CD8α (BD Pharmingen, San Diego, CA). 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
skewing in the generation of CD4 vs CD8 TCR\textsuperscript{\beta}\textsuperscript{high} cells in this system (Fig. 1B). The blockade in positive selection seems to occur at early stages in the process, because both TCR\textsuperscript{\beta}\textsuperscript{high} and TCR\textsuperscript{\beta}\textsuperscript{low} HSA\textsuperscript{low} 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 TCR\textsuperscript{\beta}\textsuperscript{high} cells in the KSR-infected thymocytes, we performed time course experiments. As shown in Fig. 2, the decrease in the generation of TCR\textsuperscript{\beta}\textsuperscript{high} 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 (MEK\textsubscript{KD}+/+) (35). E15.5 fetal thymocytes from C57BL/6 and MHC\textsuperscript{c} 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 TCR\textsuperscript{\beta}\textsuperscript{high} 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 TCR\textsuperscript{\beta}\textsuperscript{high} 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\textsuperscript{\beta}\textsuperscript{low-mid} DP cells for both the GFP\textsuperscript{+} and GFP\textsuperscript{−} populations. In each experiment in this system, the GFP\textsuperscript{−} cells serve as an additional internal control for the GFP\textsuperscript{+}-transduced cells within the same lobe. Fig. 1D shows a comparison of the generation of TCR\textsuperscript{\beta}\textsuperscript{high} cells in GFP\textsuperscript{+} vs GFP\textsuperscript{−} cells for thyocytes infected with KSR or vector alone (Mig) for five different experiments. Infection with the KSR retrovirus result in levels of KSR expression that correlates linearly with the intensity of the GFP staining (data not shown), and, in fetal thymocytes, results in at least 3-fold greater than that detected endogenously in average (data not shown). Altogether, these results show that overexpression of KSR results in a reduction in the number of mature αβ T cells in this rFTOC system. In contrast, we could not detect any significant

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure1}
\caption{KSR overexpression affects thymocyte development. rFTOCs overexpressing KSR and the control vector Mig were analyzed for the expression of TCR\textbeta, GFP, CD4, and CD8 following 9 days of culture. A, The numbers indicated above each quadrant represent the percentage of the TCR\textbeta\textsuperscript{high} cells in either the GFP\textsuperscript{+} or GFP\textsuperscript{−} populations. KSR infection, unlike Mig, results in a dramatic reduction in the percentage of TCR\textbeta\textsuperscript{high} cells relative to uninfected cells. B, Analysis of the CD4/CD8 profiles within the TCRβ\textsuperscript{high} 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 # TCRβ\textsuperscript{high}/TCRβ\textsuperscript{DP}, which represents the ratio of the number of TCR\textbeta\textsuperscript{high} cells to the number of DP cells within the TCRβ\textsuperscript{low-mid} populations. This figure shows that KSR infection reduces the fraction of mature TCR\textbeta\textsuperscript{high} cells relative to empty vector-infected or uninfected cells. Each GFP\textsuperscript{−} and GFP\textsuperscript{+} pair represents an individual lobe with the results of two different experiments analyzed at day 9 shown. Both experiments were performed using MHC\textsuperscript{c} donor thymocytes. D, Ratio of TCR\textbeta\textsuperscript{high} cells in GFP\textsuperscript{+} vs GFP\textsuperscript{−} 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 thyocytes after 6 days of rFTOC. Thyocytes were infected with empty Mig or Mig/KSR, reaggregated, and cultured for 6 days.
\end{figure}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure2}
\caption{Time course analysis of the effect of KSR on T cell development in rFTOCs. rFTOCs derived from E15.5 B6D2F1 fetal thyocytes overexpressing KSR and the control vector Mig were analyzed for the expression of TCR\textbeta, GFP, CD4, and CD8 following 6, 8, and 12 days of culture. A, The numbers indicated above each quadrant represent the percentage of TCR\textbeta\textsuperscript{high} cells in the GFP\textsuperscript{−} or GFP\textsuperscript{+} populations. At all stages of development analyzed, KSR infection results in a reduction in the percentage of TCR\textbeta\textsuperscript{high} cells. B, The CD4/CD8 profiles of the TCR\textbeta\textsuperscript{high} 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\textsuperscript{+}CD8\textsuperscript{−} cells can be observed at all time points.
\end{figure}
FIGURE 3. Infection with dMEK and KSR affects the generation of TCRβhigh cells. rFTOCs derived from C57BL/6 donor thymocytes were harvested at day 8 and analyzed for GFP and TCRβ expression. A, The numbers within each box indicate the percentage of TCRβhigh cells in the GFP− or GFP+ populations. Two different rFTOCs are shown, demonstrating that, unlike the control vector Mig, infection with dMEK or KSR reduces the fraction of TCRβhigh, mature cells. B, The CD4/CD8 distribution of TCRβhigh cells shows no significant alteration in the CD4/CD8 ratios and an accumulation of TCRβhigh DP cells in the KSR and dMEK rFTOCs. C, The ratio of total numbers of TCRβhigh 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 localization is regulated by both c-TAK and 14-3-3 (28). Mutations of S392 and I397/V401 to alanines 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 589 was mutated to methionine to generate the R589M mutant. A R589K 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 TCRβ expression in rFTOC assays, as described above. As seen previously, KSR overexpression results in a decrease in the percentage of TCRβhigh 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 TCRβhigh populations were also examined, demonstrating that as seen with TCRβ 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 correlates 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.

**Acknowledgments**

We thank L. Van Parijs, M. Therrien, M. Han, and S. Hedrick for providing reagents, as well as members of the Alberola-Illa, Baltimore, and Rothenberg laboratories for helpful discussion and technical assistance. We are grateful to T. Brummel, G. Hernandez-Hoyos, D. Leopold, J. Pomerantz, and P. Sternberg for critical comments regarding the manuscript.

**References**


