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KSR1 Modulates the Sensitivity of Mitogen-Activated Protein Kinase Pathway Activation in T Cells without Altering Fundamental System Outputs▽

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Mitogen-activated protein kinase (MAPK) cascades are evolutionarily conserved signaling pathways that regulate cell fate decisions. They generate a wide range of signal outputs, including graded and digital responses. In T cells, MAPK activation is digital in response to T-cell-receptor stimulation; however, whether other receptors on T cells that lead to MAPK activation are graded or digital is unknown. Here we evaluate MAPK activation in T cells at the single-cell level. We show that T cells responded digitally to stimulation with superantigen-loaded antigen-presenting cells, whereas they responded in a graded manner to the chemokine SDF-1, demonstrating that the system output of the MAPK module is highly plastic and determined by components upstream of the MAPK module. These findings also confirm that different MAPK system outputs are used by T cells to control discrete biological functions. Scaffold proteins are essential for proper MAPK signaling and function as they physically assemble multiple components and regulators of MAPK cascades. We found that the scaffold protein KSR1 regulated the threshold required for MAPK activation in T cells without affecting the nature of the response. We conclude that KSR1 plays a central role in determining the sensitivity of T-cell responses and is thus well positioned as a key control point.

T cells are a central component of the adaptive immune response. They are activated through the recognition of ligands by their T-cell receptors (TCR) leading to proliferation; however, T cells also have a multitude of other receptors that mediate different functions, including chemotaxis and differentiation. One of the essential signaling pathways downstream of not only the TCR but also other receptors on the surface of T cells is the canonical mitogen-activated protein kinase (MAPK) cascade, composed of Raf (MAP3K), MEK (MAP2K), and ERK (MAPK) (22). When activated, MAPK modules can generate both graded and digital outputs in vivo (11, 14) (Fig. 1). In a graded system, the pathway transmits continuous information that is proportional to the input stimulus. In contrast, the all-or-none digital output can switch between two steady states but cannot rest in intermediate states, thereby functioning as a digital switch with the low and high steady states representing "off" and "on," respectively. These different signal outputs can be used to drive discrete cell fate decisions within a single cell, a principle that is strikingly illustrated in PC12 cells, in which a graded MAPK output drives cells to proliferate, whereas a digital MAPK output directs differentiation (37).

The MAPK pathway is thought to regulate both positive and negative selection during T-cell development, with activation of the MAPK module from the Golgi correlating with positive selection and activation from the plasma membrane with negative selection (7). Recent results have demonstrated that activation of the MAPK module from the Golgi generates a graded output, whereas ERK activation from the plasma membrane is digital (16). In addition, mature T cells use a digital MAPK signal output downstream of TCR engagement to commit to T-cell activation (2). Taken together, these results suggest that T cells utilize different system outputs from the MAPK module to regulate multiple biological responses in vivo. However, whether T cells can generate multiple outputs from the MAPK cascade has not been formally assessed.

It is currently unclear how diverse MAPK system dynamics, such as graded and digital outputs, as well as different sensitivity and durations of signaling, are generated within the same cell. The use of scaffold proteins as signal-processing hubs may provide a solution to this question. Scaffold proteins act as docking platforms that bind to two or more components of the MAPK module together in a protein complex (4). There are at least two ways scaffolds can modulate the system output of MAPK cascades. First, scaffolds could set the sensitivity of the system by bringing the three kinases of the MAPK module into close proximity to increase the efficiency of signal transfer, a hypothesis supported by in silico modeling studies (24, 25). Second, scaffolds could change the fundamental system output of the MAPK module. In an elegant series of experiments, Lim and coworkers engineered scaffold-specific feedback loops by regulating recruitment of positive and negative regulators to the yeast MAPK scaffold protein Ste5 (3). The resulting syn-

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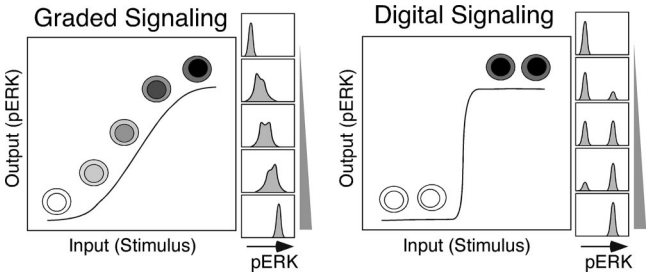

FIG. 1. Graded versus digital signaling. Hypothetical curves representing the relationship between input (stimuli) and output (pERK) at the single-cell level for graded compared to digital signaling. On the right of each curve are hypothetical flow-cytometric histograms depicting increasing pERK levels in relation to increasing stimuli for the two systems. In a graded system, the pathway transmits continuous information that is proportional to the input stimulus. In contrast, the all-or-nothing digital output can switch between two steady states but cannot rest in intermediate states, thereby functioning as a digital switch with the low and high steady states representing "off" and "on," respectively (11). These different signal outputs can be used to drive discrete cell fate decisions within a single cell (37).

thetic circuits yielded diverse MAPK outputs, providing the first critical proof-of-principle experiments that scaffold proteins themselves can be used to rewire MAPK modules to generate different outputs (3). Recent data revealed that the yeast scaffold protein Ste5 converts the inherent switch-like signal output of the yeast mating MAPK module into a graded output, confirming that scaffolds are indeed used to modulate MAPK system output in vivo (42). Since Ste5 is expressed only in yeast, it is important determine whether mammalian MAPK scaffolds perform a similar role given the lack of homology between Ste5 and mammalian scaffold proteins.

The best-characterized mammalian MAPK scaffold protein is Kinase Suppressor of Ras (KSR). It binds to Raf, MEK, and ERK to facilitate ERK activation at the plasma membrane (31, 34). Genetic and biochemical studies in nematodes, flies, and mammals confirm that KSR1 is essential for proper MAPK signal transmission in vivo (34). KSR1 binds to protein phosphatase 2A (17, 32) and casein kinase 2 (35), positive regulators of Raf activity (17, 32, 35). KSR1 is also regulated by a positive feed-forward loop from Ras through IMP (28). Thus, KSR1 coordinates multiple MAPK positive regulatory loops, placing KSR1 in a prime position to regulate MAPK system sensitivity, output, or both.

We examined here activation of the MAPK cascade in single T cells. We show that engagement of TCR with superantigen (SAg) results in a digital ERK response, whereas stimulation through a G-protein coupled receptor (GPCR), CXCR4, generates a graded ERK output, formally demonstrating that T cells generate multiple system outputs from the MAPK module. We then show that the MAPK scaffold KSR1 does not rewire the MAPK pathway to generate digital or graded outputs. Instead, the primary function of KSR1 is to modulate MAPK system sensitivity. Finally, we demonstrate that KSR1 protein levels are regulated during T-cell activation, revealing KSR1 as a likely control point for T-cell responsiveness. These findings have important implications for our understanding of T-cell regulation by the MAPK pathway in vivo.

MATERIALS AND METHODS

Cell culture, plasmids, and antibodies. Jurkat T cells and Daudi B cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum, glutamine, penicillin, and streptomycin. Goat anti-mouse immunoglobulin G and goat anti-human immunoglobulin G conjugated to phycoerythrin or biotin were from Jackson Immunoresearch Laboratories and streptavidin-TC was from BD Biosciences. The KSR1 knockdown cells and pMX-KSR1-green fluorescent protein (GFP) fusion were previously reported (13). In some cases, GFP fused to a catalytically inactivated HDAC6 was used as a GFP fusion control for its similarity in size to KSR1-GFP, but no observable difference was seen with GFP only. MSCV-KSR1-FLAG-IRES-GFP and its variants were a generous gift from R. Lewis (University of Nebraska Medical Center). Anti-phospho-ERK, anti-FLAG (M2), and antitubulin antibodies were from Sigma. For technical reasons, the data for Fig. 3B use pERK from Cell Signaling (Rb polyclonal). Anti-human TCR (c305) was produced as ascitic fluid. Anti-mCD3 (2C11) and anti-mCD28 (37.51) antibodies are from BD Biosciences and Southern Biotech, respectively. Anti-KSR1 and anti-cRaf are from Transduction Labs, and anti-MEK1 and anti-ERK2 are from Santa Cruz Biotechnology, Inc. Staphylococcal enterotoxin E (SEE) was from Toxin Technologies, and SDF-1 was kindly provided by D. Fremont (Washington University).

Transfection and intracellular staining. Jurkat T cells were electroporated (250 V, 960 μF) with the indicated DNA. Prior to use, cells were rested in serum-free medium 30 min prior to stimulation. Cells were then fixed in ice-cold methanol for at least 20 min. In cells expressing GFP, cells were fixed in 3% paraformaldehyde for 20 min prior to permeabilization in ice-cold methanol. Cells were then resuspended in blocking medium (1% bovine serum albumin and 2 mM EDTA in phosphate-buffered saline) and incubated with anti-pERK antibody (1:750 of ascites), followed by the appropriate secondary antibody. The data were then acquired by a fluorescence-activated cell sorting scan flow cytometer and analyzed by using FlowJo software. During analysis, antigen-presenting cells (APCs) stained with anti-human immunoglobulin were gated out.

Primary T-cell isolation and chemotaxis assay. T cells were isolated according to the protocol from the EasySep negative selection $CD4^+$ T-cell kit (StemCell Technologies), and purity was measured by flow cytometry with cells stained for $CD4^+$ and $Thy1^+$. Chemotaxis assays were performed as previously described (43). Briefly, cells were rested for 1 h at 37 degrees in migration medium (RPMI 1640 with 0.5% bovine serum albumin and 20 mM HEPES [pH 7.0]). A total of 5×10^5 cells were placed in the 6.5-mm Transwell insert with a pore size of 5.0 μm (Costar) and allowed to migrate for 4 h. The migration index was determined by counting the number of cells that migrated through divided by the number of cells that migrated through in the absence of chemokine.

RESULTS

The MAPK module generates both digital and graded outputs in T cells. To begin our studies, we wanted to develop a robust, single-cell system for studying different MAPK system outputs in T cells. We chose Jurkat T cells due to their ease of transfection and well-characterized ERK activation profile in response to TCR activation. Because the peptide specificity of the Jurkat TCR is unknown, we stimulated the cells with the SAg, SEE, bound to APCs (15). This physiologic stimulation resulted in rapid and synchronous ERK activation, with most cells remaining active for more than 60 min (Fig. 2). To characterize whether ERK activation in response to SEE results in a graded or digital output in Jurkat cells, we activated cells with serial dilutions of SEE and measured the active ERK output at a single cell level by flow cytometry using an antibody that specifically recognizes the active, biphosphorylated form of ERK (pERK). Importantly, SEE was diluted prior to incubating with APCs to ensure that all stimulations had the same number of APCs. Strikingly, we observed only two states of ERK activation in response to SEE stimulation at 3 min. As the SEE levels increased, the number of cells that became activated also increased in a dose-dependent fashion without individual cells displaying intermediate levels of ERK activation (Fig. 3A). Since this type of bimodal output response is the

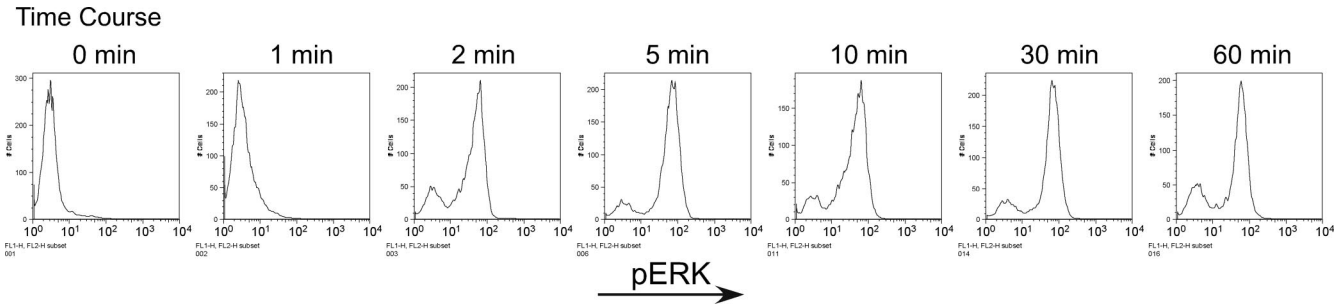

FIG. 2. Activation of the MAPK pathway in Jurkat T cells is rapid and sustained. Jurkat T cells were stimulated with SEE-coated (1 μg/ml) APCs for the indicated time points. Cells were then fixed and stained for pERK and analyzed by flow cytometry. Samples were also stained with anti-human immunoglobulin to exclude APCs during analysis.

hallmark of digital systems, we conclude that ERK activation in Jurkat T cells by the SAg, SEE, is digital. To investigate whether stimulation of the TCR alone is sufficient to generate the digital response, we stimulated cells with an anti-TCR antibody. This response was also digital; however, anti-TCR stimulation resulted in a slightly broader "on" peak (Fig. 3B). This difference could be due to other receptor-ligand interactions between the T-cell and the APC that help to enforce the "digitalness" of the system since T cells are only activated by APCs in physiologic settings.

To determine whether all pathways leading to the activation of ERK in Jurkat T cells results in a digital response, we stimulated cells with serially diluted amounts of the chemokine, SDF-1. SDF-1 induces ERK activation in cells by triggering the GPCR, CXCR4 (12, 39). In contrast to SEE activation, as SDF-1 levels increased, individual cells showed intermediate levels of pERK with the degree of ERK activation corresponding to SDF-1 input (Fig. 3C). These results confirm that T cells display a graded ERK output in response to SDF-1. Interestingly, the pharmacologic activator of the MAPK pathway, phorbol myristate acetate (PMA), behaved in a graded manner as well (Fig. 3D). In combination with the SEE stimulations above, these data show that T cells can generate both digital and graded MAPK system outputs in vivo. We conclude that the system output from the MAPK module in T cells is highly plastic and suggest that the type of output generated from the module is determined by signals emanating from the initiating receptor.

It is important to note that while ERK activation in response to SDF-1 and PMA, although distinct from the strong bimodal character of TCR signaling, did not increase in a perfectly linear fashion as the stimulus increased. We suspect that true analog outputs are rare in biological circuits that are used to generate digital as well as graded outputs, since some degree of nonlinearity is necessary for digital signaling to occur (11, 18). Rather, weakly switch-like outputs are used to functionally mimic analog circuits, as is the case for SDF-1 and PMA activation of the MAPK module in T cells. For this reason we specifically use the word "graded" instead of "analog" throughout.

Reducing KSR1 levels lowers the sensitivity of the digital MAPK system without changing its fundamental system output. In mammals, there are two KSR isoforms, KSR1 and KSR2. We focused our study on KSR1, since we have been unable to detect KSR2 expression in mouse or human T cells. It is therefore unlikely that KSR2 plays any role in T cells. In addition, we previously demonstrated that KSR1-deficient T cells show reduced levels of ERK activation when stimulated with antibodies to the TCR or PMA (31), confirming that KSR1 is essential for proper ERK activation in T cells. However, these experiments were done at the population level and therefore cannot discriminate whether the reduced ERK output was due to a change in system sensitivity or whether the system output was fundamentally changed within individual cells.

First, we sought to formally determine the role of KSR1 in modulating MAPK system output. We utilized a previously reported KSR1 knockdown Jurkat cell line to assess how reducing the level of KSR1 affected the MAPK system output in response to SEE activation at a single cell level (13). KSR1 knockdown cells still exhibited digital ERK activation after TCR engagement. This was demonstrated by the bimodal distribution of pERK staining (Fig. 4A) and the absence of any cells with intermediate staining. In addition, the intensity of pERK staining for both the "off" and the "on" populations was similar to the controls even though KSR1 levels were significantly decreased. These results show that KSR1 is not responsible for generating the digital MAPK system output.

KSR1 depletion did, however, significantly modulate the sensitivity of the system to SEE input. In the KSR1 knockdown line, significantly fewer cells were activated at any given input relative to control cells (Fig. 4A). Thus, high KSR1 expression increases the probability of a cell activating for a given input, whereas the loss of KSR1 expression decreases probability of activation. These findings are entirely consistent with early work showing that optimal KSR expression increases the probability of the all-or-nothing cell fate decision of *Xenopus* oocyte maturation (6). In combination with our data, the two studies clearly demonstrate that KSR1 does not affect the digital nature of the MAPK response. Instead, these results show that in digital MAPK systems the scaffold protein KSR1 functions to lower the threshold for ERK activation.

Reducing KSR1 levels lower the sensitivity of the graded MAPK system without changing its fundamental system response. We next explored the possibility that KSR1 might be involved in facilitating graded ERK responses. ERK activation occurs only after the phosphorylation of a threonine and tyrosine residue by its upstream activator, MEK. Experimental

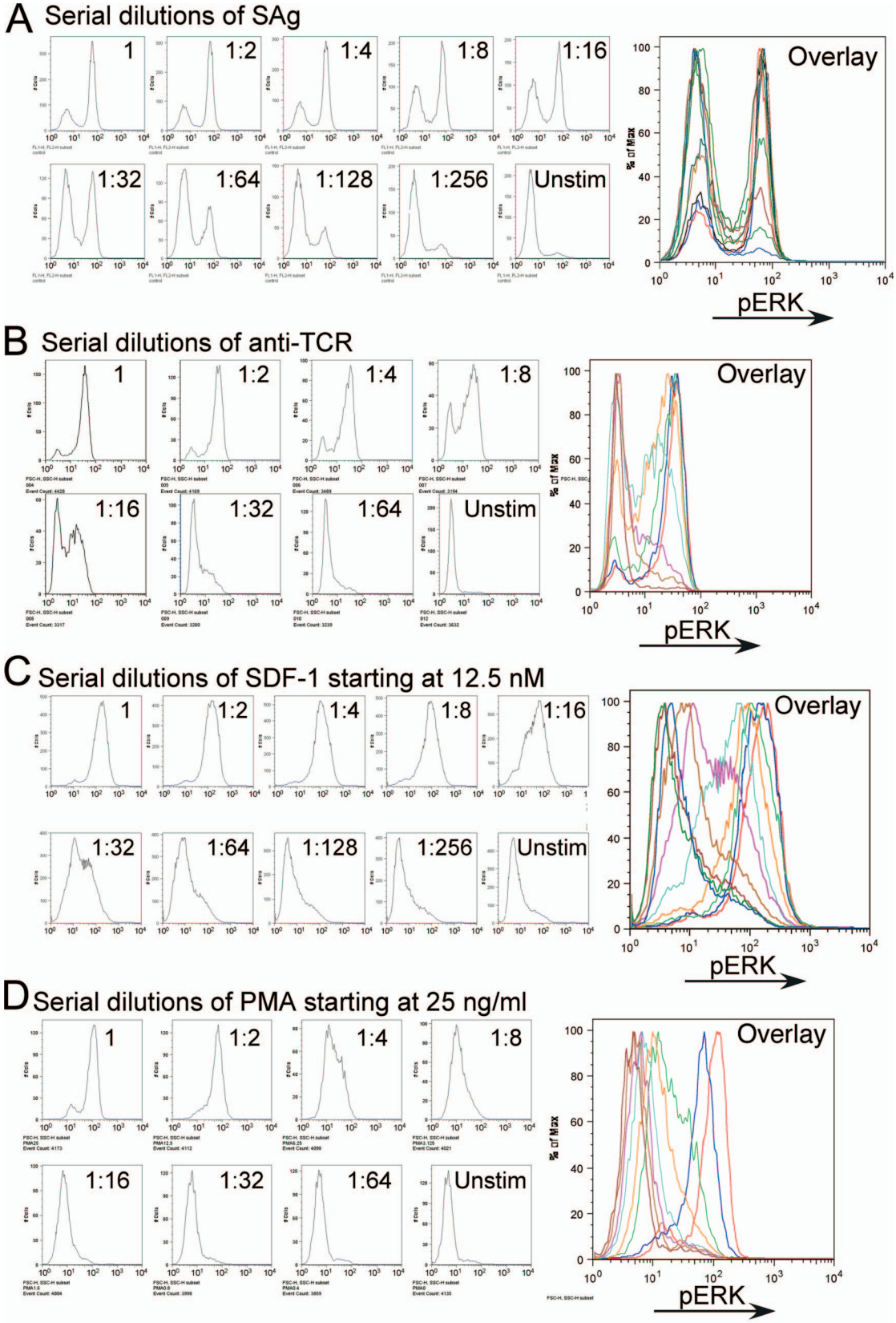

FIG. 3. Jurkat T cells can generate both digital and graded responses. (A) SEE was serially diluted twofold starting at 250 ng/ml prior to incubation with APCs. Cells were then stimulated for 3 min, fixed, and stained with an anti-pERK monoclonal antibody and the appropriate secondary antibody, followed by analysis by flow cytometry. (B) Jurkat T cells were stimulated with twofold serially diluted concentrations of anti-TCR monoclonal antibody starting at a 1:500 dilution of ascitic fluid for 3 min and analyzed with an anti-pERK polyclonal antibody. (C) Jurkat T cells were stimulated with twofold serially diluted concentrations of SDF-1 starting at 12.5 nM for 3 min and analyzed as described for panel A. (D) Cells were stimulated with 2-fold serial dilutions starting at 25 ng of PMA/ml for 3 min and analyzed as described for panel A.

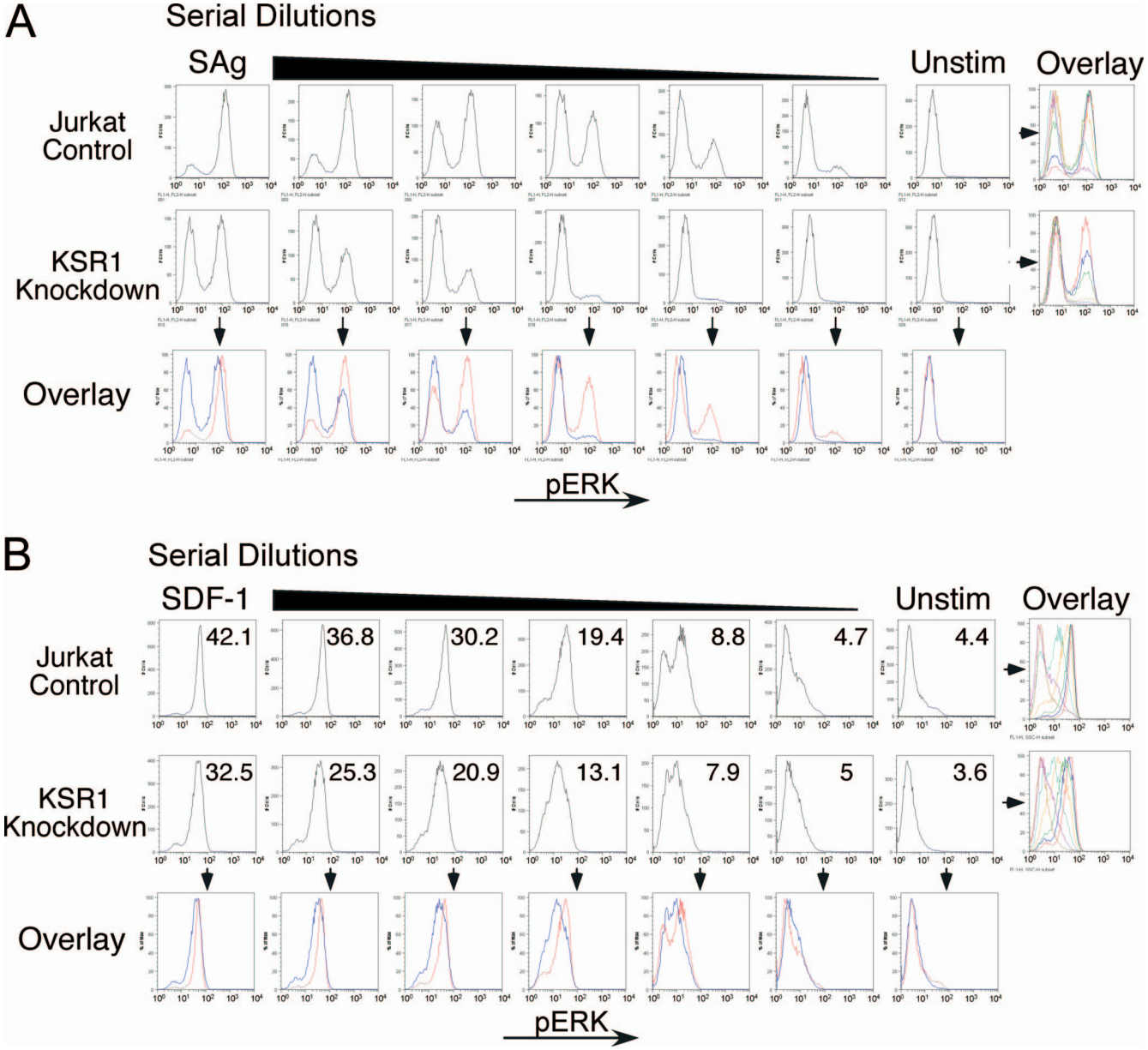

FIG. 4. KSR1 knockdown increases the threshold required to activate ERK at a per-cell basis. (A) Control T cells or KSR1 knockdown cells were stimulated for 3 min with serially diluted SEE-coated APCs followed by staining with an anti-pERK monoclonal antibody and the appropriate secondary antibody. Cells were then analyzed by flow cytometry. Shown are fourfold serial dilutions starting at 250 ng of SEE/ml. (B) Control T cells or KSR1 knockdown cells were stimulated for 3 min with serially diluted SDF-1, followed by staining and analysis as described for panel A. Shown are twofold serial dilutions starting at 5 nM SDF-1. Inset numbers represent the mean fluorescence intensity. The panels on the right show the overlay of all of the stimulation points for the individual control and KSR1 knockdown cell lines. The lower panels show the overlay between control and KSR1 knockdown cells for each individual stimulation point.

data demonstrate that when ERK and MEK are in solution, each of the phosphorylation events occurs independently, a mechanism termed "distributive phosphorylation" (5, 10). Kinetic modeling demonstrates that the requirement for two independent events to activate ERK could contribute to a digital response. (27). Since KSR1 couples MEK and ERK together and therefore the two phosphorylation events (4), KSR1 may be predicted to enhance the graded response. Recent results in yeast studies provide strong experimental support for this hypothesis (42); however, the generality of this model has not been tested.

We therefore tested whether suppression of KSR1 in Jurkat cells affected the graded response of ERK to SDF-1. SDF-1 stimulation continued to produce a graded ERK output in KSR1-depleted Jurkat T cells (Fig. 4B). KSR1 depletion did, however, reduce the sensitivity of the system, since KSR1-depleted cells generated less pERK in response to SDF-1 stimulation relative to control cells at various doses of SDF-1. To

ensure these results were due to the reduction in KSR1 expression and not an off-target effect of the KSR1 knock-down, we repeated these experiments with mouse embryo fibroblasts derived from wild-type or KSR1 knockout mice (31) using epidermal growth factor as the input signal. Precisely the same results were obtained in the MEF KSR1 knockout system as for the Jurkat KSR1 knockdown system; the absence of KSR1 reduced the sensitivity of the MAPK response but did not change the graded system output (data not shown). In combination, these results argue that, in contrast to the yeast Ste5 scaffold protein, mammalian KSR1 does not enhance graded signaling from the MAPK pathway.

Increasing KSR1 expression levels do not reconfigure a digital or graded MAPK system. We next tested whether increasing expression of KSR1 rewired the digital SEE system to generate a graded output. Although it has been previously published that ectopic expression of MAPK scaffolds inhibit MAPK activation (23, 44), none of these studies have carefully examined this effect on digital or graded systems at a single cell level. Jurkat cells were transfected with a KSR1-GFP fusion to allow us to examine the effect of increasing amounts of KSR1 on digital SAg-induced ERK activation. Strikingly, when the levels of KSR1 reached a critical level, ERK activation was completely inhibited (Fig. 5A). Importantly, the SEE system retained a digital system output as KSR1 levels increased. Moreover, KSR1 inhibition of the SEE system was digital, whereas KSR1 inhibition of the SDF-1 system was graded, mirroring the activation responses (Fig. 5A). This is clearly shown when a narrow GFP gate is drawn at the inflection point between on and off states so as to maintain roughly equal numbers of cells that are on and off. Cells exist at an intermediate state when stimulated with SDF-1, whereas with SEE the cells display a bimodal distribution. It is important to note that while the width of the GFP gates are identical for the two stimuli, the gate for cells stimulated with SDF-1 had to be shifted slight to the right to maintain roughly equal numbers of cells that are on and off, reflecting that slightly higher levels of KSR1 are required to inhibit SDF-1-induced ERK activation compared to SEE-induced ERK activation (Fig. 5A). In combination, these data strongly argue that KSR1 does not reconfigure the MAPK module from a digital to a graded output. It is interesting that increases in KSR1 levels did not appreciably increase the sensitivity of the digital SEE system or the graded SDF-1 system in Jurkat cells (Fig. 5B and C). This result indicates that KSR1 is expressed at close to the optimal levels for signal transduction in this cell line. From these experiments, we conclude that KSR1 does not engineer the MAPK module to generate different system outputs but functions solely to determine the sensitivity of the systems in which it is used.

KSR1 scaffold function is critically dependent on MEK binding. High-level expression of scaffold proteins inhibits signaling cascades by blocking the productive interactions of pathway constituents (6, 19, 24, 36, 41). Early studies characterizing the role of KSR1 demonstrated that MEK-KSR1 binding is crucial for KSR1 function (30, 44). However, more recent results suggest the role of KSR1 in regulating the MAPK pathway is more complicated than originally thought. For example, KSR1 participates in Raf activation (35), which may explain why the loss of MEK binding did not inhibit the ability of KSR1 (C809Y) to alter the biological actions of oncogenic Ras (20). To confirm that KSR1 functions as a scaffold in T cells, we examined the inhibitory ability of ectopically expressed mutant KSR1 proteins with compromised scaffold function. As seen previously, ectopic expression of wild-type KSR1 completely inhibited ERK activation once the inhibitory level of KSR1 expression was reached (Fig. 5A and 6B). To confirm that KSR1 functions as a scaffold in T cells, we tested the mutant KSR1 protein that cannot bind MEK (C809Y) (30, 44) for its ability to inhibit MAPK signaling. Western blotting of immunoprecipitated KSR1 wild type and KSR1 mutant (C809Y) confirmed that the mutant protein does not bind MEK even under TCR-stimulated conditions (Fig. 6A). The KSR1 (C809Y) mutant failed to inhibit ERK activation at any level of expression, confirming that the scaffolding the MAPK module is a crucial function of KSR1 in T cells (Fig. 6B).

If the role of scaffolds, such as KSR1, is to simply link MEK to ERK, the inhibitory effect due to overexpression of KSR1 should in theory be a dose dependent, graded inhibition due to a "dilution" effect as previously modeled (24). To determine how the dose-dependent inhibition of ERK activation due to a competitive inhibitor at a single cell level would appear, we ectopically expressed a kinase-dead version of MEK that functions as a dominant negative. Its inhibition of ERK is much more graded at high expression levels, which is consistent with the idea of a competitive inhibitor (Fig. 6C). Also, ectopic expression of another scaffold that links MEK and ERK, MP-1 (38), did not induce the same inhibitory effect as KSR1 overexpression (data not shown). Clearly, the mechanism of inhibition induced by ectopic expression of KSR1 is not as simple as originally thought and will require further investigation into possible mechanisms involving cooperative binding and feedback loops.

KSR1 is important for proper activation of the MAPK pathway and biological output in primary T cells. To confirm our findings in a more physiologic system, we directly examined the effect of the loss of KSR1 expression on ERK activation in primary T cells isolated from KSR1-deficient mice. Primary $CD4^+$ T cells isolated from wild-type and KSR1 knockout mice were subjected to stimulation with either anti-TCR antibodies or SDF-1. As previously reported, the absence of KSR1 severely impaired TCR-induced ERK activation (Fig. 7A) (31). Strikingly, SDF-1 stimulation also demonstrated a severe ERK activation defect in KSR1-deficient primary T cells, confirming our results obtained with Jurkat T cells (Fig. 7A). Although the crucial role of KSR1 during TCR mediated T-cell activation is well established (31), it is unknown how the loss of KSR1 affects SDF-1-mediated T-cell function. To directly address this question, we performed chemotaxis assays on primary T cells isolated from wild-type and KSR1 knockout mice. In line with their impaired ERK activation, KSR1-deficient T cells exhibited a substantial defect in their ability to migrate toward SDF-1 (Fig. 7B). From these results, we conclude that KSR1 does play a physiologically important role in regulating the sensitivity of ERK activation to SDF-1.

KSR1 expression is carefully regulated following T-cell activation. To investigate our hypothesis that KSR1 plays a central role in setting ERK activation thresholds, we examined whether KSR1 levels were actively regulated during T-cell responses. Naive versus primed T cells are thought to have dif-

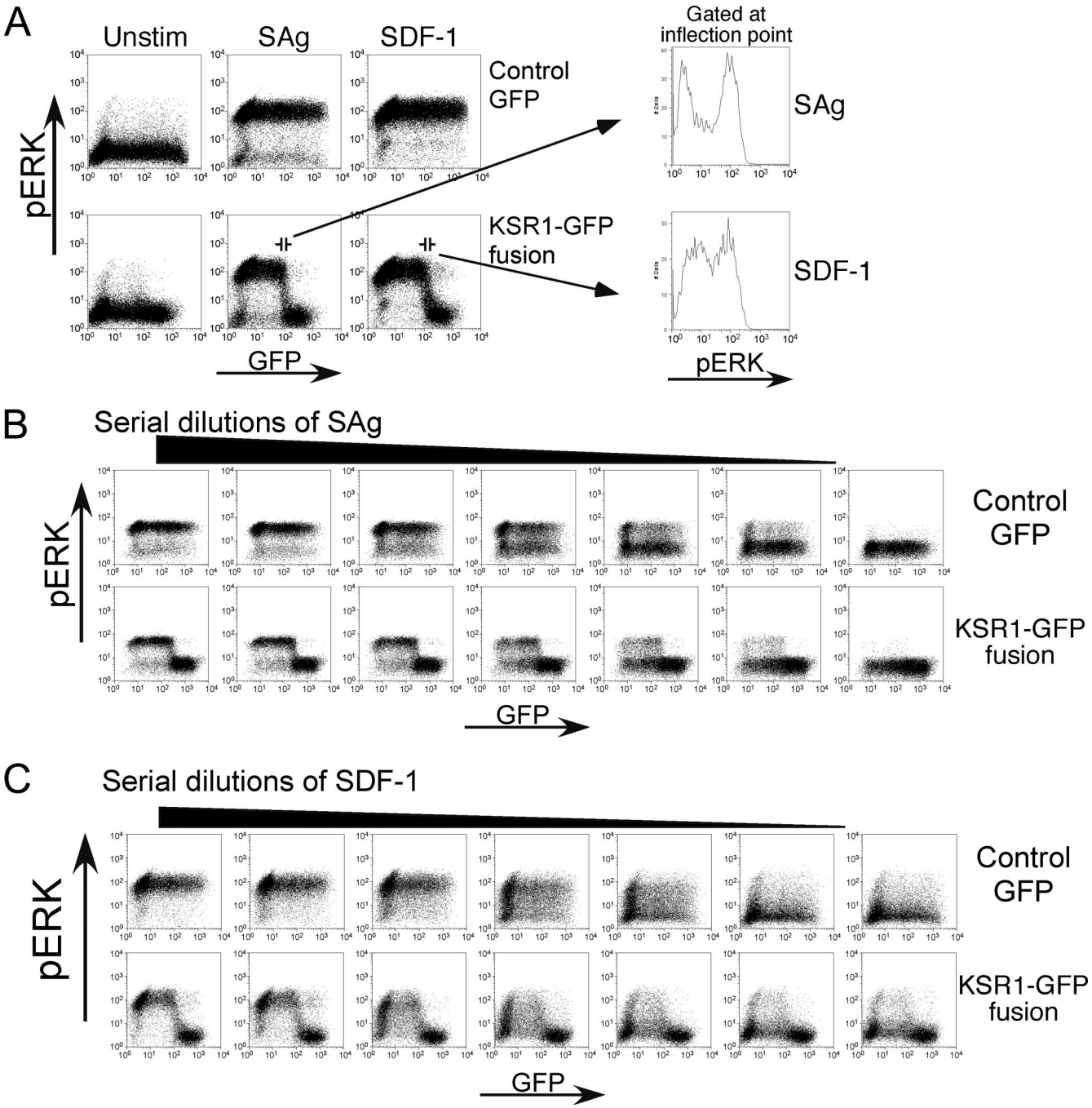

FIG. 5. Overexpression of KSR1 inhibits ERK activation. (A) Jurkat T cells were transfected with either a control GFP or KSR1-GFP fusion and, 18 h later, the cells were stimulated for 3 min with either SEE-coated (1 μg/ml) APCs or 1 μM SDF-1. Cells were then stained with anti-pERK and analyzed by flow cytometry. A narrow gate was drawn to show the cells at the inflection point of low to high pERK staining. Note that the gate is slightly shifted to the right in the SDF-1 stimulation to maintain approximately equal numbers of low and high cells in the histogram. (B) Control GFP or KSR1-GFP transfected cells were stimulated for 3 min with serially diluted SEE-coated APCs, followed by staining for pERK. Cells were then analyzed by flow cytometry. Shown are fourfold serial dilutions starting at 250 ng of SEE/ml. (C) Jurkat T cells were transfected with either a control GFP or KSR1-GFP fusion and, 18 h later, the cells were stimulated for 3 min with twofold serially diluted SDF-1 starting at 16 nM. Cells were then stained for pERK and analyzed by flow cytometry.

ferent activation requirement for proliferation and cytokine release (reviewed in reference 1). To determine whether KSR1 levels were playing a role in this differential activation requirement, we isolated $CD4^+$ T cells from mice and compared KSR1 levels from freshly isolated cells to cells that had been stimulated with anti-CD3 and anti-CD28 for 5 days. We also compared KSR1 expression levels relative to the MAPK components Raf, MEK, and ERK in these cells. Strikingly, KSR1 expression dramatically decreased as T cells differentiated relative to the components of the MAPK module (Fig. 8). These

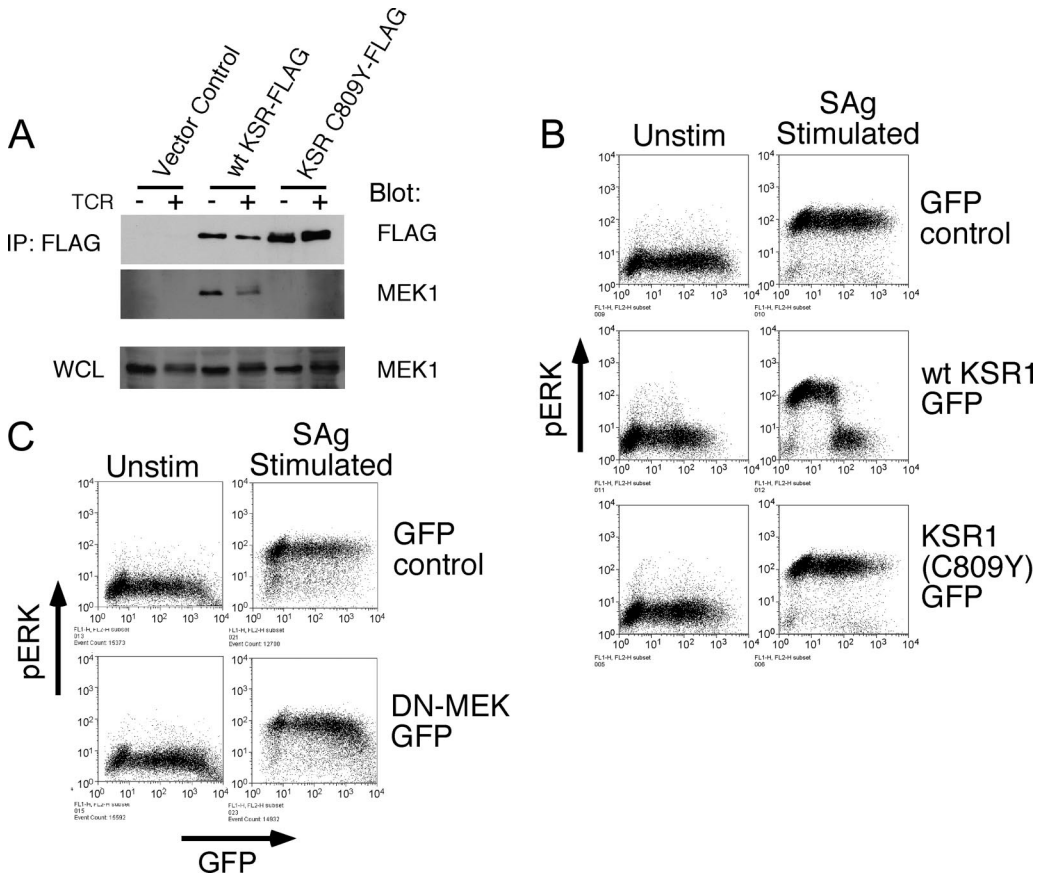

FIG. 6. KSR1 inhibition requires MEK binding. (A) Jurkat T cells were transfected with vector control, wild-type (wt) KSR1-FLAG, or KSR (C809Y)-FLAG and then left unstimulated or stimulated for 3 min with anti-TCR. Cells were then lysed and immunoprecipitated with an anti-FLAG monoclonal antibody. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and visualized by immunoblotting with anti-FLAG and anti-MEK1 antibodies. (B) Jurkat T cells were transfected with control GFP, KSR1-IRES-GFP, or KSR (C809Y)-IRES-GFP. After 18 h, the cells were stimulated for 3 min, as before, with SEE-coated APC (1 μg/ml). Cells were fixed and stained as described above. (C) Jurkat T cells were transfected with control GFP or a dominant-negative MEK (DN-MEK). Cells were then stimulated with SEE-coated APC (1 μg/ml), fixed, and stained as described above.

data reveal that KSR1 expression level is indeed regulated during T-cell activation relative to the components of the MAPK module.

DISCUSSION

The three-tiered architecture of MAPK modules has been highly conserved throughout eukaryotic evolution, confirming the utility of this circuit configuration in biology (22). It has become increasingly clear that MAPK modules are highly plastic, generating multiple types of signal outputs that are used by cells to regulate divergent biological functions (14). Here we provide direct experimental support that this paradigm holds true for T cells. Activation of the MAPK module by the TCR generates a digital response, whereas activation of the module by a GPCR is graded. These distinct MAPK outputs are functionally suited for their respective T-cell biological functions. Since T-cell activation by the TCR is a discrete cell fate decision, a digital output from the MAPK module is appropriate to drive this decision-making process (2). In the case of a chemokine response, cells are interpreting a chemokine gradient to determine in which direction to migrate. In this instance, the appropriate MAPK output is a graded one, with the amount of output increasing as the cell moves into areas with increasingly higher concentrations of chemokines.

Our finding that output from the MAPK is highly plastic in T cells suggests that signals emanating from the initiating receptor are used to set the type of MAPK response. Since scaffolds can potentially integrate positive- and negative-feedback loops to shape the output of kinase cascades (3), we tested whether differential usage of KSR1, the best-characterized mammalian ERK scaffold, was responsible for the differences in TCR versus GPCR MAPK signal output. Our findings do not support a specific role of KSR1 in determining the system output of the MAPK module. Instead, KSR1 enhanced the efficiency of ERK activation in both graded and digital systems, confirming recent predictions based on computational models of scaffold function (25).

The distributive, two-step phosphorylation of ERK is an important contributor to digital signal output (10, 18). Tethering MEK and ERK in a scaffold complex is predicted to suppress distributive phosphorylation and thereby inhibit digital output to enhance graded signaling (5), a prediction con-

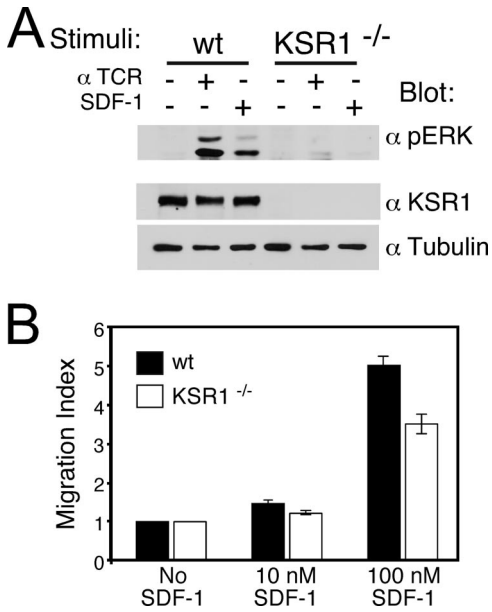

FIG. 7. KSR1-deficient primary T cells fail to activate ERK in response to TCR or SDF-1. (A) Primary $CD4^+$ T cells were isolated from wild-type (wt) or KSR1 knockout mice to >85% purity and stimulated with either an anti-TCR antibody or 100 nM SDF-1 for 3 min. Cells were then lysed, and ERK activation was measured by pERK blotting. (B) Chemotaxis of purified primary $CD4^+$ T cells from either wild-type (wt) or KSR1 knockout mice was measured by a Transwell migration assay. The migration index is a measure of the number of cells that migrated into the bottom chamber, in the presence of the indicated SDF-1 concentration, divided by the number of cells that migrated with no chemokine. The bars represent the averages, and the error bars indicate the standard deviations of samples evaluated in triplicate.

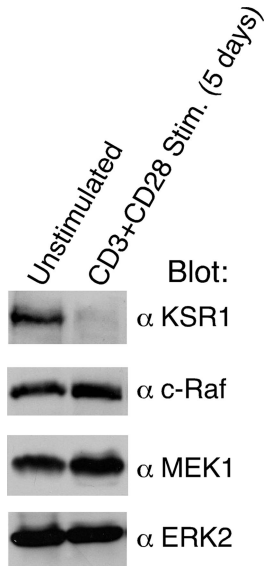

FIG. 8. KSR1 levels are regulated in primary T cells after stimulation. Primary $CD4^+$ T cells were isolated from wt mice and stimulated with plate-bound anti-CD3 and anti-CD28 antibody for 5 days. Cells were then lysed, and proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. KSR1, c-Raf, MEK1, and ERK2 were visualized by immunoblotting with their respective antibodies.

firmed recently in the yeast MAPK scaffold Ste5; however, it is important to note that the readouts in this system were transcriptionally regulated (42). Given these results, it was surprising that KSR1 failed to enhance graded MAPK signaling. Our data reveal that different scaffold proteins have qualitatively different effects on MAPK signaling; thus, it may not be possible to make generalizations about how scaffolds modulate MAPK signaling. Rather, the regulatory functions of individual scaffold proteins need to be determined empirically. A challenge for the future will be to determine the biochemical basis of digital and graded responses. It seems likely that a variety of different factors, including scaffold proteins, localization within the cell, positive and negative feedback, and the complexity of signaling pathways, will each play roles in determining the final character of a signaling response.

A central question in T-cell biology is how TCR responses are tuned during T-cell development, activation, and differentiation in the periphery (9). The threshold for discrimination between pMHC ligands changes during T-cell maturation. As T cells mature, they maintain the same sensitivity for foreign agonist ligands while losing the capacity to respond to weak self-pMHC complexes (8, 26). Recent experimental results suggest that T cells may also modify their pMHC discrimination threshold in the periphery by using endogenous pMHC ligands to boost T-cell response to agonist pMHC ligands (21, 40). These combined observations show that TCR responsiveness is not set for each TCR but is fine-tuned depending on the differentiation state of the T cell. The molecular mechanisms underlying the tuneability of the TCR are not well understood. Our results show that KSR1 occupies a prime position from which to regulate the plasticity of T-cell responses, with changes in KSR1 expression level being able to modulate both the threshold of TCR activation and the rate of chemotaxis. Consistent with this role, we show that KSR1 expression levels are tightly regulated during T-cell activation, as naive T cells express significantly higher levels of KSR1 protein relative to their primed counterparts. These data support the previous observation that KSR1 is required for proper naive T-cell differentiation into TH1 and TH2 cells but not for cytokine production after restimulation of fully differentiated TH1 and TH2 cells (31). Also, in support of this model, other groups have reported that KSR1 function can be modulated by caspase-dependent cleavage of KSR1 or changes in KSR1 protein half-life (29, 33). Future work will explore changes in KSR1 expression during thymocyte development and T-cell differentiation. Also, examining KSR1 levels in anergic or tolerized T cells could provide insight into the biology of these cells.

In conclusion, we have demonstrated the MAPK module is highly plastic in T cells, displaying fundamentally different system outputs and sensitivities. We go on to show that the scaffold protein, KSR1, modulates MAPK system sensitivity but not system output. We also reveal that KSR1 expression level is carefully regulated during T-cell maturation. These findings identify KSR1 as a likely control point used to fine-tune T-cell responses during T-cell activation and differentiation, suggesting KSR1 as a key regulator of T-cell function and plasticity in vivo.

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