The mitogen-activated protein kinase scaffold KSR1 is required for recruitment of extracellular signal-regulated kinase to the immunological synapse

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The Mitogen-Activated Protein Kinase Scaffold KSR1 Is Required for Recruitment of Extracellular Signal-Regulated Kinase to the Immunological Synapse

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KSR1 is a mitogen-activated protein (MAP) kinase scaffold that enhances the activation of the MAP kinase extracellular signal-regulated kinase (ERK). The function of KSR1 in NK cell function is not known. Here we show that KSR1 is required for efficient NK-mediated cytosis and polarization of cytolytic granules. Single-cell analysis showed that ERK is activated in an all-or-none fashion in both wild-type and KSR1-deficient cells. In the absence of KSR1, however, the efficiency of ERK activation is attenuated. Imaging studies showed that KSR1 is recruited to the immunological synapse during T-cell activation and that membrane recruitment of KSR1 is required for recruitment of active ERK to the synapse.

Kinase suppressor of Ras was originally identified in Drosophila melanogaster (53) and Caenorhabditis elegans (19, 32, 52) as a positive regulator of the extracellular signal-regulated kinase (ERK) mitogen-activated protein (MAP) kinase signaling pathway. It is thought to function as a MAP kinase scaffold because it can bind to Raf, MEK, and ERK (18, 19, 27, 28, 44, 59). While the exact function of KSR is unknown, preassembling the three components of the ERK MAP kinase cascade could function to enhance the efficiency of ERK activation, potentially regulate the subcellular location of ERK activation, and promote access to specific subcellular substrates (16, 45, 46).

While only one isoform of KSR is expressed in Drosophila (53), two KSR isoforms have been identified in C. elegans (19, 32, 52) and most higher organisms. They are referred to as KSR1 and KSR2 (32, 43). While KSR1 mRNA and protein are detectable in a wide variety of cells and tissues, including brain, thymus, and muscle (10, 11, 29), little is known about the expression pattern of KSR2.

We previously reported the phenotype of KSR1-deficient mice (30). These mice are born at Mendelian ratios and develop without any obvious defects. Using gel filtration, we showed that KSR1 promotes the formation of large signaling complexes containing KSR1, Raf, MEK, and ERK (30). Using both primary T cells stimulated with antibodies to the T-cell receptor as well as fibroblasts stimulated with growth factors, we showed that KSR1-deficient cells exhibit an attenuation of ERK activation with defects in cell proliferation.

Here we explored the role of KSR1 in NK cell-mediated cytosis. The killing of a target cell by a cytolytic T cell or NK cell is a complicated process that involves cell polarization with microtubule-dependent movement of cytolytic granules to an area that is proximal to the contact surface or immunological synapse (7, 33, 34, 48–50, 54). A variety of different signaling molecules are also involved, including calcium (23), phosphatidylinositol-3,4,5-triphosphate (13, 17), and activation of the ERK MAP kinase (6, 42, 56). Recently, the recruitment of activated ERK to the immunological synapse (IS) has been shown to be a feature of successful killing of a target by cytotoxic T lymphocytes (58).

How active ERK is recruited to the synapse is not known. Since KSR1 is known to be recruited to the plasma membrane by Ras activation (24), and since the immunological synapse is one of the major sites of Ras activation (26, 41), it seemed plausible to test the hypothesis that KSR1 recruitment to the plasma membrane functions to recruit ERK to the immunological synapse and facilitate its activation. We found that KSR1 was recruited to the immunological synapse and that KSR1 appeared to be required for the localization of active ERK at the contact site. As KSR1-deficient cells exhibit a defect in killing, this suggests that KSR1 recruitment to the synapse may be important in the cytolytic killing of target cells.

MATERIALS AND METHODS

Mice. KSR1-deficient mice (KSR1−/−) have been described previously (30). All mice were housed under specific-pathogen-free conditions in the Washington University animal facilities in accordance with institutional guidelines.

Cell cultures and antibodies. Jurkat E6.1 T cells, Daudi lymphoma B cells, YAC-1 lymphoma cells, human K562 erythroleukemia cells, and RMAs and RMAs-Rae1 cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS). Human interleukin-2 (hIL-2)–dependent cell line NK92 cells (15) were grown in RPMI 1640 medium supplemented with 10% FBS and 100 U/ml of hIL-2. Mouse NK cells were purified by DX5+ magnetic-activated cell sorting enrichment (Miltenyi) and grown in hIL-2-containing medium (5). Polyoma viral anti-Grb2, rabbit anti-ERK2, rabbit anti-KSR1, and mouse anti-Lck were obtained from Santa Cruz Biotechnology. Polyclonal rabbit anti-phospho-ERK [pERK1/2 (Thr202/Tyr204)] and rabbit anti-phospho-
MAP/CDK substrates (PXSP) were obtained from Cell Signaling Technology. Monoclonal anti-ERK kinase (diphosphorylated ERK1/2) and mouse anti-α-tubulin were purchased from Sigma. Fluorescein isothiocyanate-labeled Cd3ε and phycoerythrin (PE)-NK1.1-labeled antibody were obtained from BD Biosciences.

Generation of DNA constructs. Murine KSR1 (mKSR1) full-length cDNA was subcloned into a pEF1-N1 vector (Clontech). After EcoRI and NotI digestion, mKSR1-YFP was cloned into a pMX retrovirus vector (31). The C359 and C362 mutants in the CA3 domain of mKSR1 (CCSS mutant) were generated using PCR site-directed mutagenesis (Stratagene). The primers used for C359W were 5′-ATT TTG GGT GGC AAG AAC CAC TGC AGG-3′ and 5′-CCT GCA GGA TTG CCT CAC GCC AAA AAT C-3′; for C362W they were 5′-GGT AAG AAC AAA CAC AGC TTA TTA AAA TGC CAT AAC-3′ and 5′-GTT ACG GCA TTT AAT TCC GCT GTG TTT GCT CTT C-3′. The integrity of all constructs was confirmed by automated sequencing.

Retroviral transduction. The Phoenix amphotropic retroviral packaging cell line was kindly provided by Gary Nolans. After transfection using Lipofectamine 2000 (Life Technologies), cells were transferred to 32°C to allow accumulation of virus in the supernatant. Virus-containing supernatant was harvested at 24 and 48 h after transfection and filtered through 0.45-μm syringe filters (Millipore). Jurkat cells were incubated with viral supernatant in the presence of 8 μg of Polybrene (Sigma) and then centrifuged at 900 g for 5 min at room temperature (37°C) on a Beckton Dickinson FACSVantage SE at the Flow Cytometry Core Facility (Dept. of Pathology and Immunology, Washington University, St. Louis, MO).

RNA interference and lentivirus production. KSR1 small hairpin RNA (shRNA) and luciferase shRNA (control) constructs were generated using the multifunctional lentivirus system (pFLRu lentivector; provided by Y. Feng and G. D. Longmore). To generate human ksr1 shRNA fragments, two sequences corresponding to nucleotides 1507 to 1530 and 2139 to 2157 were selected. Primers (sequence 1 forward, GTG GAA AGG ACG AAA CAC CGC CTA and sequence 1 reverse, TCT GCC ATG TCT TGC TTC GCT TTT GCT CTT CAC) were generated, digested with complementary counterparts and annealed by PCR to obtain shRNA fragments. Joint PCR was carried out by using human U6 promoter forward primer (ACG GAA TTC TAG AAC CCC AGT AGA AAC AGG CGC AGG) and shRNA reverse primer, and mixed template (1 μl of purified human U6 promoter and 2 μl of purified shRNA fragment). The PCR products were purified, digested with XhoI/XbaI, and subcloned into pPLRu lentivector for pFLRu (kSR1-shRNA). To reconstitute kSR1 expression, mKSR1-YFP wild-type and CCSS mutant were subcloned into the pFLRu-kSR1#1-shRNA vector to create pFLRu-kSR1#1-shRNA-mKSR1(WT)-YFP and pFLRu-(kSR1#1-shRNA)-mKSR1(CCSS)-YFP, respectively. The integrity of all constructs was verified by automated sequencing. For lentivirus production, subconfluent cultures of 293T cells were incubated for 24 h with Polybrene (Sigma) and then centrifuged at 900 g for 5 min at room temperature. Cells were re-suspended in ice-cold lysis buffer (0.1 M Tris base, 1 M NaCl, 1% SDS) and sonicated for 30 min at room temperature. After centrifugation, proteins from cell lysates were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and analyzed by immunoblotting. KSR1 protein bands were detected and quantified by immunoblotting using goat anti-HRP-conjugated anti-mouse antibody (Jackson ImmunoResearch Laboratories, Inc.) for 30 min at room temperature. After washing, the blots were incubated with horseradish peroxidase. KSR1 expression was monitored by binding with a specific antibody (anti-Rae1 antibody conjugated to biotin; provided by Marina Cellia) followed by the appropriate secondary antibody. The ratio between RMAs and RMAs-Rae1 cells from CFSE-labeled cells was determined by flow cytometry.

Cell conjugation assay. Target cells were loaded with 10 μM CFSE for 15 min at 37°C. NK cells from WT or kSR1−/− mice were stained with PE-labeled CD3ε antibody (Biosynthesis Biotechnology, St. Louis, MO) and mixed with an equal amount of Daudi B cells preloaded with or without staphylococcal enterotoxin E (SEE). After conjugation, cells were pelleted and resuspended on poly-l-lysine-coated glass slides for 5 min at 37°C, fixed, permeabilized, and stained as described above. To quantitate the recruitment of KSR1-YFP or YFP to the contact site, boxes were drawn at the contact area between the effector and target cells, at the cytosol, and in a background area outside the cell by using the Image J software program (NIH). The relative recruitment index (RRI) was calculated as follows: (mean fluorescence intensity [MFI] at synapse − background)/MFI at regions in the cytosol − background. For each experiment, the percentage of Jurkat cells with an RRI of more than 1.1 was calculated. For quantification of permeability translocation to the cell-cell contact area, the ratio of MFI at the contact area versus an equivalent in the cytosol was calculated and a ratio of more than 1.1 was scored as protein accumulation. At least 50 conjugates were examined for each experiment, and three different experiments were performed.

Cytotoxicity assays. Cytotoxic activity of mouse NK cells was tested against YAC-1 or RMAs or RMAs-Rae1 target cells using standard 4-h 51Cr release assays (5). Where indicated, NK cells were preincubated with 10 μM specific MEK inhibitor (U0126; Calbiochem) at 37°C for 30 min. In all experiments, spontaneous release did not exceed 10% of maximum release.

CFSE labeling and in vivo NK killing assay. The in vivo NK cell cytotoxicity experiments were performed essentially as previously described (3). RMAs and RMAs-Rae1 cells (105) were labeled with 1 μM (low peak) or 10 μM (high peak) CFSE (Molecular Probes) for 15 min at 37°C in RPMI 1640 medium supplemented with 5% FBS. Labeling was blocked with 1x (vol/vol) BSA, and cells were washed several times with RPMI complete medium. CFSE-loaded cells were counted, mixed at a 1:1 ratio, and injected intraperitoneally (8 × 104 to 10 × 104 cells/mouse in a 300-μl volume) in WT and kSR1−/− mice. A small sample of injection mix was acquired at the zero time point to record the ratio between RMAs and RMAs-Rae1 cells. At 24 h after injection, cells were recovered by peritoneal lavage. Cells were stained with anti-mouse CD3ε (145-2C11) antibody (BioLegend) and anti-mouse immunoglobulin (Jackson ImmunoResearch Laboratories, Inc.) for 30 min at room temperature. After washing, cells were incubated with Cy3-conjugated goat anti-mouse immunoglobulin (Jackson ImmunoResearch Laboratories, Inc.) for 30 min at room temperature. After washing, cells were analyzed by flow cytometry.

Flow cytometric measurement of intracellular ERK activation. T cells transduced with the indicated lentivectors were mixed with SEE-pulsed Daudi B cells at a 1:1 ratio, spun at 350 × g for 10 s, and placed at 37°C for 5 min. T-cell antigen-presenting cell (APC) conjugates were then separated with ice-cold PBS–2.5 mM EDTA and fixed with 4% paraformaldehyde for 10 min on ice. Cells were permeabilized with 90% methanol for 30 min at −20°C, washed with PBS containing 3% fetal bovine serum, and incubated with mouse anti-ϕ ERK for 45 min at room temperature. After washing, cells were incubated with phycoerythrin-labeled F(ab′)2 anti-mouse immunoglobulin (Jackson ImmunoResearch Laboratories, Inc.) for 30 min at room temperature. KSR1 expression was measured by flow cytometry after gating for YFP-positive T cells and analyzed using FlowJo.

Cell stimulation, immunoprecipitation, and immunoblotting. Jurkat cells transduced with the indicated lentivectors were starved for 1 h in RPMI 1640. Daudi cells were loaded with 100 ng/ml of SEE (Toxin Technology, Inc.) for 30 min before mixing 2:1 (T cells:B cells) with T cells in RPMI medium. Cells were gently centrifuged for 30 s and placed at 37°C for the indicated times. After stimulation, the pellet was resuspended in ice-cold lysis buffer (0.1 M Tris base, 140 mM NaCl, 1 mM EDTA, 1% NP-40, 1 mg/ml BSA, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, and 50 mM sodium fluoride). After centrifugation, proteins from cell lysates were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and analyzed by immunoblotting with the indicated primary antibodies followed by incubation with anti-mouse IgG or anti-rabbit IgG conjugated to horseradish peroxidase. To quantitate the level of KSR1, whole-cell lysates from Jurkat or NK92 cells transduced with the indicated lentivector were resolved by SDS-PAGE. KSR1 protein bands were detected and quantified by immunoblotting with the Odyssey system (Li-Cor). NK92 cells (5 × 105/sample) transduced with the indicated lentivectors, after sorting, were labeled with IL-2 starved for 4 h in RPMI 1640 containing 5% FBS. Cells were incubated with an equal number of K562 target cells at 37°C for the indicated times. Cells were resuspended in ice-cold lysis buffer and were centrifuged at 1000 g for 5 min. Cell lysates were analyzed by immunoblotting with antibodies specific for phosphorylated ERK. In the immunoprecipitation experiments, nucleus-free supernatant was incubated with 2 μg/ml of monoclonal anti-Lck at 4°C for 60 min and then incubated with protein A-Sepharose beads (Pharmacia) at 4°C for 90 min. After washing, Lck

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immunoprecipitates were resolved by SDS-PAGE, transferred to a membrane, and analyzed by immunoblotting with the indicated antibodies.

Statistics. Statistical analyses were performed using a paired Student’s t test. Differences that were statistically significant are noted in the figures below.

RESULTS

KSR1 is required for NK lytic activity. Previously we showed that thymic and peripheral T-cell populations in KSR1-/- mice were similar to wild type (30). To determine the role of KSR1 in NK cell development, we measured the numbers of NK cells by using antibodies to CD3 and NK1.1 in the spleens of wild-type and KSR1-/- mice (Fig. 1A). Flow cytometric analysis showed that NK cells (NK1.1+ CD3- cells) were normally represented in spleens of KSR1-/- mice (Fig. 1B). We also observed that KSR1 deficiency did not affect the numbers of NKT cells (NK1.1+ CD3+). Altogether, these data suggest that NK cell development is normal in KSR1-/- mice.

We then tested the role of KSR1 in NK cell killing. Splenic NK cells from wild-type and KSR1-deficient mice were purified and NK lytic activity was tested by incubation with YAC-1 target cells. While wild-type NK cells efficiently killed YAC-1 cells (Fig. 2A), there was a significant reduction of killing when using KSR1-deficient NK cells.

Since YAC-1 cell recognition is complex and involves several different receptors (5), we also tested NK lytic activity mediated by the NK receptor NKG2D. For these experiments we used RMAs cells transfected with the mouse NKG2D ligand Rae1ε (RMAs-Rae1ε) as targets. NK cells from KSR1-deficient mice showed a significant reduction in cytolytic activity (Fig. 2B) that was specific to NKG2D, as there was no killing of RMAs cells lacking Rae1ε expression. Importantly, the reduction of NK cell cytotoxicity was not mediated by the decreased cell-cell adhesion, since the absence of KSR1 did not affect the ability of NK cells to form conjugates with the indicated target cells (Fig. 2C).

We confirmed the NK cell killing defect in vivo by injecting wild-type and KSR1-deficient mice with RMAs and RMAs-Rae1ε cells and monitoring tumor growth as previously described (3). In this system, elimination of RMAs-Rae1ε cells is mediated by NK cells in an NKG2D-dependent manner. RMAs and RMAs-Rae1ε cells (10^7) were distinguished by either 1 μM (low staining) or 10 μM (high staining) CFSE, respectively. Tumor cells were mixed at a 1:1 ratio and injected intraperitoneally (8 x 10^6 to 10 x 10^6 cells/mouse in a 300-μl volume). A sample was measured before injection to document the starting ratio between RMAs and RMAs-Rae1ε cells (Fig. 2D and G). Twenty-four hours after injection, cells were recovered by peritoneal lavage and stained with anti-Rae1ε and the ratio between RMAs and RMAs-Rae1ε cells in the CFSE-labeled cells was determined by flow cytometry. As expected (12), RMAs-Rae1ε cells were preferentially eliminated in wild-type mice (Fig. 2E and G). In contrast, elimination of RMAs-Rae1ε cells in KSR1-/- mice was impaired (Fig. 2F and G). This demonstrates that KSR1 is required to mediate NK cell lytic activity in vivo.

NKG2D-induced lytic granule polarization is impaired in KSR1-/- mice. NK killing of the target cells is mediated by the polarized release of lytic granules (13, 34). Since NK lytic activity was impaired in the absence of KSR1, we investigated whether KSR1 was required for lytic granule polarization. Purified NK cells from wild-type and KSR1-/- mice were incubated with Lysotracker to label lytic granules and then imaged before and after conjugate formation with YAC-1 cells. Prior to conjugation, lytic granules were randomly distributed in the cytosol (Fig. 3A). After interaction with YAC-1 cells, lytic granules of wild-type NK cells moved to a location near the site of contact with the target cell (Fig. 3A and C). This was spe-
specific, as the polarized movement of lytic granules was inhibited by using inhibitors of phosphatidylinositol 3 kinase (data not shown). While the ability of KSR1-deficient NK cells to form conjugates with YAC-1 cells was not affected, lytic granule polarization was significantly reduced (Fig. 3B and C). These results suggest that KSR1 is also important in lytic granule polarization and that defects in granule polarization may be responsible for defects in cytolytic killing.
PERK recruitment into the NK IS is impaired in KSR1−/− mice. Recently it has been reported that pERK is recruited to the immunological synapse of CD8+ T cells (58). Since it is postulated that the subcellular localization of signaling molecules is mediated by scaffold molecules (18, 39, 45), we wondered whether KSR1 might be involved in ERK localization to the IS.

We tested whether KSR1 plays a role in pERK localization by first imaging pERK localization using NK cells from KSR1-deficient mice. Purified NK cells from wild-type and KSR1−/− mice were conjugated with YAC-1 cells. Cells were stained with antibodies to pERK and analyzed by confocal microscopy. While pERK was detectable in both wild-type and KSR1-deficient NK cells, pERK localization at the synapses was infrequent in KSR1-deficient NK cells compared to wild-type cells (Fig. 4A and B).

So that we could dissect the mechanism of KSR1 function, we attempted to replicate these findings using Jurkat cells where KSR1 expression was suppressed using lentiviruses expressing two different KSR1-specific shRNAs. Bulk sorting of green fluorescent protein-positive cells showed that both shRNAs resulted in over 70% inhibition of expression (Fig. 4C). pERK recruitment to the synapse was then analyzed by forming conjugates between Jurkat cells and superantigen-coated APCs (Fig. 4D and E). While pERK was easily detected at the IS of conjugates formed using wild-type Jurkat cells (Fig. 4D, upper panels, and E), suppression of KSR1 expression significantly impaired the recruitment of pERK to the IS (Fig. 4D, lower panels, and E). Similar results were obtained when the same shRNAs were used in the human NK cell line NK92 (data not shown).

Consistent with results from KSR-deficient T cells (30), suppression of KSR1 expression in the Jurkat cells attenuated ERK activation (Fig. 5A). This suggested that our inability to detect pERK at the synapse could be due to a generalized defect in ERK activation. This seemed unlikely, as strong staining with the pERK antibody was easily detected in some of the KSR1-deficient cells (Fig. 4D). Germain and coworkers have demonstrated that ERK activation by the T-cell receptor is all-or-none in individual CD8+ T cells (1). What they found was that as the strength of T-cell receptor (TCR) signaling increases, there is not a graded increase in ERK activation. Rather, at the individual cell level, ERK is either fully activated in cells or not activated at all. We, therefore, hypothesized that in the absence of KSR1, cells could still be activated but that the total number of activated cells was lower. To confirm this, we used flow cytometry to compare ERK activation in control versus KSR1 shRNA-treated cells. As we expected, KSR1 shRNA cells were able to activate ERK to levels similar to wild-type cells but the number of cells that were activated was much lower (Fig. 5B). This suggests that KSR1 functions to increase the sensitivity of TCR-mediated activation of ERK. In addition, it suggests that the lack of pERK at the synapse is not due to a generalized defect in ERK activation and supports the hypothesis that KSR1 is required for the synapse localization of pERK.

KSR1 recruitment is required for pERK accumulation into the immunological synapse. To determine whether KSR1 itself is recruited to the IS, Jurkat T cells were transduced with a construct encoding KSR1 fused to YFP (KSR1-YFP). After conjugation with superantigen-coated APCs, KSR1-YFP was easily detected at the IS (Fig. 6A, lower panel, and B). As a control, YFP by itself was distributed homogenously throughout Jurkat cells with or without stimulation by SEE (Fig. 6C). The KSR1-YFP recruitment was specific to T-cell activation, as conjugation with APCs in the absence of superantigen did not result in cell death (Fig. 6D). The KSR1-YFP recruitment was specific to T-cell activation, as conjugation with APCs in the absence of superantigen did not result in cell death (Fig. 6D).
not result in any detectable KSR1 recruitment to the synapse (Fig. 6A, upper panel, and B).

After Ras activation, KSR1 is recruited to the plasma membrane via its CA3 domain (24). To verify that synapse recruitment of KSR1 is responsible for pERK localization in the synapse, we rescued KSR1-deficient Jurkat cells with either a CA3-mutated KSR1-YFP construct or a wild-type KSR1-YFP fusion. The CA3 domain has two conserved cysteine residues that can be mutated to disrupt the structure of the domain and its ability to bind to membranes (60). Cell sorting was used to isolate a population of cells with similar expression levels (Fig. 7A). Since high-level expression of KSR1 is known to inhibit ERK activation (20, 21), we first verified that the level of KSR1 expression in the cells that we isolated was able to restore ERK activation. Flow cytometric analysis showed that the level of wild-type KSR1 was sufficient to reconstitute ERK activation.
KSR1-mediated ERK activation.
The requirement for active nological synapse.
ment to the IS is required for pERK localization at the immu-
the IS is mediated by its CA3 domain and that KSR1 recruit-
Together, these results demonstrate that KSR1 recruitment to
experiments showed that the wild-type KSR1-YFP was re-
KSR1-YFP could rescue pERK localization at the IS. Imaging
in the absence of membrane recruitment (Fig. 7B).
Interestingly, the CA3 mutant was able to partially rescue ERK
activation, suggesting that KSR1 can facilitate ERK activation
in the absence of membrane recruitment (Fig. 7B).
We next tested whether cells expressing the CA3-mutated
KSR1-YFP could rescue pERK localization at the IS. Imaging
experiments showed that the wild-type KSR1-YFP was re-
cruited to the synapse and was able to rescue pERK localiza-
tion at the IS. In contrast, the CA3 mutant was not recruited to
the IS, nor was pERK detectable at the IS (Fig. 7C and D).
Together, these results demonstrate that KSR1 recruitment to
the IS is mediated by its CA3 domain and that KSR1 recruit-
ment to the IS is required for pERK localization at the immu-
nological synapse.
Phosphorylation of the Lck PXSP motif is regulated by
KSR1-mediated ERK activation. The requirement for active
ERK recruitment to the IS suggests that it is required for the
phosphorylation of proteins that are present in the synapse.

Since Lck is a known substrate for ERK during T-cell activa-
tion (47, 55, 57), we tested whether KSR1 was required for Lck
phosphorylation. A KSR1-specific shRNA-expressing lentivi-
rus was used to inhibit endogenous KSR1 expression in a
human NK cell line (Fig. 8A). We confirmed that suppression
of KSR1 reduced ERK activation in the human NK cell line
after stimulation with target cells (K562 cells) (Fig. 8B). Lck
immunoprecipitates were prepared from both wild-type and
KSR1 shRNA-expressing cells and blotted with an antibody
that recognizes ERK phosphorylation sites (PXSP). The in-
duction of Lck phosphorylation after target cell incubation was
reduced in KSR1 shRNA-treated NK cells compared to wild-
type cells (Fig. 8C). This supports the hypothesis that KSR1
recruitment of ERK facilitates the phosphorylation of ERK
substrates at the synapse.

DISCUSSION

Here we examined the role of KSR1 on the cytolytic func-
tion of NK cells and found that KSR1-deficient NK cells ex-
hibit a defect in killing. The defect appeared to be related to an
inability to polarize cytolytic granules. Since pERK recruit-
ment to the immunological synapse was recently reported dur-
ing the activation of CD8 T cells (58), and because ERK
activation is required for killing (6, 56), we explored the hy-
pothesis that pERK recruitment to the synapse might be facili-
tated by KSR1. Indeed, we found that in KSR1-deficient T
cells, pERK recruitment to the immunological synapse was
defective.

KSR1 is thought to function as a scaffold for the Ras/MAP
kinase pathway (18, 27, 28, 44). This scaffold molecule regu-
lates the intensity and duration of growth factor-induced ERK
activation, where it presumably facilitates the interaction be-
 tween active Ras and Raf-1 (18, 28, 35). More recent data
suggest that KSR may have additional roles facilitating phos-
phorylation of the ERK activation loop of Raf (9). Previously,
we showed that in KSR1-deficient T cells the activation of ERK
was still detectable but highly attenuated (30). We interpreted
this to mean that KSR1 is required for the efficient activation
of ERK.

In this study, we analyzed the ERK activation defect in more
detail. We previously used immunoblotting to measure ERK
activation (10, 30). This method, because it relies on the lysis
of millions of cells, averages the biochemical changes that occur
at a specific moment in time. Using such a method, an atten-
uation of ERK activation could be due to attenuation of ERK
activation in all cells or reflect a defect in ERK activation in
some but not others. Using flow cytometry to analyze ERK
activation at a single-cell level, we were surprised to find that
in KSR1-deficient cells, the defect of ERK activation only
affected some but not all cells. A small fraction of KSR1-
deficient cells showed levels of ERK activation that are similar
to wild-type cells.
Previous work had suggested that ERK activation in CD8 T
cells is stochastic, that it is an all-or-none process (1). A weak
stimulus results in only a few cells that are fully activated and
as the strength of a stimulus is increased, more and more cells show full ERK activation. In support of this idea, we found that the attenuation of ERK activation seen in KSR1-deficient cells is due to decreased numbers of activated cells, suggesting that KSR1 functions by lowering the threshold stimulus required for the stochastic activation of ERK. We speculate that by helping to recruit the Raf/MEK/ERK module to active Ras, KSR1 may function to enhance the activation of the pathway (22).

It is intriguing to speculate that recruitment of KSR1 and the ERK MAP kinase cascade to the immunological synapse may have functions in addition to simply facilitating ERK activation. By holding active ERK at the immunological synapse, KSR1 may function to allow ERK to phosphorylate specific substrates at the plasma membrane essential for T-cell function. For example, it has been proposed that ERK phosphorylation of Lck may play an important role in facilitating a positive feedback loop that is important for enhancing T-cell activation (47). Our immunoprecipitation data indicated that ERK-dependent phosphorylation of the PXSP motif in Lck was diminished after KSR1 suppression, supporting the role of KSR1 on ERK substrate phosphorylation into the synapse. Other important substrates at the immunological synapse include stathmin, a molecule that plays a key role in helping to regulate microtubule polymerization (4). It seems possible that the granule polarization defect seen in the KSR1-deficient cells

FIG. 6. KSR1 is recruited into the immunological synapse. (A) Representative differential interference contrast, YFP, and Cy3 fluorescence images of Jurkat T cells expressing KSR1-YFP after conjugation with Daudi B cells loaded with or without SEE (100 ng/ml). In the absence of SEE (-SEE), ERK (red) is not phosphorylated and KSR1 (green) is not recruited into the contact site. In the far right panel, the location of pERK is shown in false color. Bar, 5 μm. (B) KSR1 and pERK accumulation at the contact site was quantitated from three independent experiments with at least 50 conjugates. Data are represented as the average (± standard error of the mean) of conjugates with an RRI of >1.1 (see Materials and Methods). (C) Representative differential interference contrast, YFP, and Cy3 fluorescence images of Jurkat T cells expressing YFP conjugated with Daudi B cells and stimulated as for panel A. Images are representative of two independent experiments with at least 30 conjugates.
is due to defects in ERK phosphorylation of critical substrates at the immunological synapse.

The localization of the MAP kinase cascade at different sites in the cell has been suggested to play an important role in T-cell biology (26). While it was originally thought that Ras activation of the MAP kinase cascade could only be initiated at the plasma membrane, it has now become clear that different Ras isoforms are localized and activated at distinct intracellular membranes (2, 8, 37). At steady state, while K-Ras is mainly localized to the plasma membrane and N-Ras and H-Ras are...
mainly localized to the Golgi apparatus (36). Philips and co-workers showed that TCR stimulation alone resulted mainly in Golgi complex activation of Ras, while costimulation with anti-LFA-1 allowed for plasma membrane and Golgi complex activation of Ras, while costimulation with anti-LFA-1 allowed for plasma membrane and Golgi complex activation of Ras, while costimulation with anti-LFA-1 allowed for plasma membrane and Golgi complex activation of Ras, while costimulation with anti-LFA-1 allowed for plasma membrane and Golgi complex activation of Ras, while costimulation with anti-LFA-1 allowed for plasma membrane and Golgi complex activation of Ras, while costimulation with anti-LFA-1 allowed for plasma membrane and Golgi complex activation of Ras, while costimulation with anti-LFA-1 allowed for plasma membrane and Golgi complex activation of Ras. In these studies, the localization of Ras signaling is important in the activation of loca-

...ntunately, little is known about the expression pattern of KSR2. Probe sets for KSR2 are available on commercial microarrays for both human and mouse, but there are no data documenting any level of expression in any tissue. In our own hands, we have been unable to detect KSR2 message using a variety of different methods in any lymphoid or myeloid compartment.

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FIG. 8. ERK phosphorylation of Lck is facilitated by KSR1 in NK cells. (A) Inhibition of KSR1 expression after KSR1-specific shRNA transduction in the human NK92 cell line (3 × 106 cells/lane). Immuno- blotting was performed with antibodies to KSR1 and Grb2. (B) Defective ERK activation in KSR1 knockdown NK92 cells. NK92 cells were stimulated with target cells (K562) for the indicated times (in minutes) and analyzed for pERK1/2 by Western blotting. Blotting with α-tubulin (α-tub) was used to demonstrate equal loading. (C) Serine phosphorylation of the Lck PXSP motif is facilitated by KSR1. Control and KSR1 shRNA-expressing NK92 cells were incubated with K562 cells as described for panel B. Lck immunoprecipitates were prepared at the indicated times (in minutes) and were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and probed with a phospho-specific antibody to the sequence PXSpSP. The membrane was then stripped and reprobed with monoclonal anti-Lck to confirm equal loading of Lck.