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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 cytolsis 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 cytolsis. 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-trisphosphate (13, 17), and activation of the ERK MAP kinase (6, 42, 56).

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-Rae1e cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS). Human interleukin-2 (hIL-2)-dependent cell line NK22 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). Polyclonal rabbit 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-
MAPK/CDK substrates (PXS) were obtained from Cell Signaling Technology. Monoclonal anti-MAP kinase (diphosphorylated ERK1/2) and mouse anti-erbB2 antibody 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 pMEF-PWI vector (Clontech). After EcoRI and NotI digestion, mKSR1-YFP was cloned into a PMX retrovirus vector (31). The C59 and C62 mutants in the CA3 domain of mKSR1 (CCSS mutant) were generated using PCR site-directed mutagenesis (Stratagene). The primers used for C59/62 were 5′-ATT TTG GCC GGT TAT AAG AAC CAC TGC AGC-3′ and 5′-CTC GCA GGA TTT TAA CCT GCT GTG TTT GCT CTT CAC-3′; for C62/63 they were 5′-GGT AAG AAC AAA CAC AGC AGG TTA AAA TGC CAT AAC-3′ and 5′-GGT ATG AGC TTA CAA CCT GCT GTG TTT GCT CTT CAC-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 Nolan. After transfection with Lipofectamine 2000 (Life Technologies), cells were transfected 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/ml Polybrene (Sigma) and then centrifuged at 900 g for 5 min at 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.

KSR1 small hairpin RNA

\[\text{GAA TTC TAG AAC CCC AGT GGA AAG ACG CGC AG} \]

shRNA reverse

\[\text{GGA CAA TTT TAA CCT GCT GTG TTT GCT CTT CAC-3'} \]

Forward

\[\text{ATA GGG TAT AAG AAC AAA CAC AGG TTA AAA TGC CAT AAC-3'} \]

Retroviral transduction.

J. Neumeister

\[\text{1507-1530} \]

Retroviral transduction.

\[\text{2139-2157} \]

Retroviral transduction.

\[\text{5-1507} \]

Retroviral transduction.

\[\text{5-2139} \]

Retroviral transduction.

\[\text{5-1530} \]

Retroviral transduction.

\[\text{5-2157} \]

Retroviral transduction.

\[\text{KSR1} \]

Retroviral transduction.

\[\text{CCSS mutant} \]

Retroviral transduction.

\[\text{mKSR1(CCSS)-YFP} \]

Retroviral transduction.

\[\text{mKSR1(YFP)-KSR1#1-shRNA} \]

Retroviral transduction.

\[\text{mKSR1(YFP)-KSR1#1-shRNA} \]

Retroviral transduction.

\[\text{mKSR1(YFP)-KSR1#1-shRNA} \]

Retroviral transduction.

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Retroviral transduction.

\[\text{mKSR1(YFP)-KSR1#1-shRNA} \]

Retroviral transduction.

\[\text{mKSR1(YFP)-KSR1#1-shRNA} \]
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−) 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 × 10^6 to 10 × 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-
cific, 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.

FIG. 2. KSR1 is required for NK lytic activity in vitro and in vivo. (A and B) Cytotoxicity of WT and KSR1−/− NK cells was tested against YAC-1 cells (A) or RMAs and RMAs-Rae1ε target cells (B) in vitro. Purified NK cells (>96% NK1.1+ CD3ε+) were IL-2 starved in RPMI medium for 4 h before incubation with the indicated target cells. Where indicated, NK cells were preincubated with the MEK inhibitor UO126 (10 μM). Data are representative of three independent experiments. E:T ratio, effector:target ratio. (C) Conjugate formation is normal in KSR1-deficient cells. NK cells from WT or KSR1−/− mice were stained with NK1.1-PE antibody and mixed with CFSE-loaded target cells. Cells were allowed to form conjugates for 20 min at 37°C, fixed, and analyzed by flow cytometry. The bar graphs represent the percentages of NK1.1+ CFSE− double-positive cells from the total pool of NK1.1+ cells. Data are represented as averages ± standard errors of the means of at least three separate experiments. (D to G) NK killing assay in vivo. RMAs and RMAs-Rae1ε cells were labeled with different concentrations of CFSE and mixed at a 1:1 ratio. (D) An aliquot of the cell mixture was analyzed before injection (time zero). (E and F) The cell mixture was injected intraperitoneally into WT and KSR1−/− mice. Twenty-four hours after injection, cells were recovered by peritoneal lavage. Rae1ε expression was assessed by labeling with anti-Rae1ε antibody and examined by flow cytometry. The RMAs/RMAs-Rae1ε ratio was obtained by comparing high and low CFSE-labeled cells. (G) Summary of RMAs/RMAs-Rae1ε ratios in six WT and six KSR1−/− mice. Horizontal bars indicate the mean ratios.
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 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.

FIG. 4. Recruitment of pERK into the NK IS is impaired in KSR1-deficient mice. (A) Representative images showing the localization of pERK (red) in primary NK cells purified from spleens of WT and KSR1<sup>−/−</sup> mice that were conjugated with CFSE-loaded YAC-1 target cells (green). Bar, 5 μm. (B) pERK accumulation at the NK IS was quantitated by dividing the percentage of cells with pERK at the NK IS by the total number of pERK-positive cells imaged. Data represent the mean (± standard error of the mean) percentage of conjugates with an RRI (see Materials and Methods) of >1.1, from three independent experiments with at least 30 conjugates. *, P < 0.05. (C) KSR1 knockdown in Jurkat T cells. Immunoblotting results are for KSR1 and Grb2 expression in Jurkat T cells (3.5 × 10<sup>5</sup> cells/lane) transduced with the indicated shRNA. KSR1 expression levels were compared to control shRNA Jurkat T cells. (D) pERK recruitment into the contact site is impaired in KSR1 knockdown T cells. Representative differential interference contrast and Cy3 fluorescence images are shown for shRNA-expressing Jurkat T cells conjugated with Daudi B cells preloaded with 100 ng/ml of superantigen. Bar, 5 μm. (E) Percentage of conjugates with pERK recruited into the synapse, as described for panel D. Quantitative analysis was done for pERK accumulation at the contact site from three independent experiments with at least 40 conjugates. Data represent the mean (± standard error of the mean) percentage of conjugates with an RRI of >1.1. *, P < 0.05.
Since Lck is a known substrate for ERK during T-cell activation (47, 55, 57), we tested whether KSR1 was required for Lck phosphorylation. A KSR1-specific shRNA-expressing lentivirus 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 induction 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 function of NK cells and found that KSR1-deficient NK cells exhibit a defect in killing. The defect appeared to be related to an inability to polarize cytolytic granules. Since pERK recruitment to the immunological synapse was recently reported during the activation of CD8\(^+\) T cells (58), and because ERK activation is required for killing (6, 56), we explored the hypothesis that pERK recruitment to the synapse might be facilitated 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 regulates the intensity and duration of growth factor-induced ERK activation to modulate a cell’s proliferative, oncogenic, and adipogenic potential (20, 21, 40). It binds to all three kinases of the ERK MAP kinase cascade, Raf-1, MEK, and ERK (19, 51, 53), and is recruited to the plasma membrane during Ras activation, where it presumably facilitates the interaction between active Ras and Raf-1 (18, 28, 35). More recent data suggest that KSR may have additional roles facilitating phosphorylation of the 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 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 coworkers showed that TCR stimulation alone resulted mainly in localized to the Golgi apparatus (36). Philips and coworkers showed that TCR stimulation alone resulted mainly in localized activation of Ras, while costimulation with anti-CD28 and KSR1 shRNA-expressing NK92 cells were incubated with K562 cells as described for panel A. 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 PXpSP. The membrane was then stripped and reprobed with monoclonal anti-Lck to confirm equal loading of Lck.

Whether KSR1 localizes to sites other than the plasma membrane is not yet known. It would be interesting if KSR1 and KSR2 were recruited to distinct membranes. Unfortunately, in these studies, the localization of active ERK was not determined. It is possible that this plays a role in localizing active ERK close to the location-specific effectors. In the present study, Ras signaling has been shown to occur on endosomes as well as in endoplasmic reticulum membranes (14, 38). Inherent in these studies is the idea that localized Ras signaling is important in the activation of location-specific effectors, but it is also possible that this plays a role in localizing active ERK close to the location-specific substrates. Unfortunately, in these studies, the localization of active ERK was not determined.

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 × 10^6 cells/lane). Immunoblotting 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 was performed with antibodies to KSR1 and Grb2. (C) Depletion of Lck loading of Lck.


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