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The MAPK Scaffold Kinase Suppressor of Ras Is Involved in ERK Activation by Stress and Proinflammatory Cytokines and Induction of Arthritis

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The MAPK ERK is required for LPS-induced TNF production by macrophages. Although the scaffold kinase suppressor of Ras (KSR)1 is required for efficient Erk activation by mitogenic stimuli, the role of KSR1 in ERK activation by inflammatory and stress stimuli is unknown. In this study, we examined the effects of KSR deficiency on ERK activation by stress stimuli and show that ERK activation by TNF, IL-1, and sorbitol is attenuated in the absence of KSR1. To determine the significance of this defect in vivo, we tested KSR-deficient mice using a passive transfer model of arthritis. We found that the induction of arthritis is impaired in the absence of KSR. Thus, KSR plays a role in ERK activation during inflammatory and stress responses both in vitro and in vivo. The Journal of Immunology, 2006, 177: 6152–6158.

The MAPKs are an evolutionarily conserved family of proteins that mediate cellular responsiveness to a variety of stimuli (1, 2). Three groups of MAPK exist, which in mammals have been identified as the ERK, the JNK, and the p38 kinases. The ERK MAPK is primarily associated with mitogenic signaling, whereas the JNK and p38 MAPKs are associated with stress and inflammatory signaling (3). The MAPKs are activated by MAPK kinase (or MAP2K). The MAP2K in turn is activated by MAPK kinase kinase (or MAP3K). For the ERK pathway, the three RAF kinases (c-Raf, A-Raf, and B-Raf) are thought to serve as the primary MAP3Ks. In contrast, JNK and p38 can be activated by many different MAP3Ks. Recently, MAP3K associated with the JNK and p38 pathways was found to be able to activate ERK. For example, the MAP3K MEKK1, an activator of p38 and JNK, activates ERK in response to osmotic stress (4). Additionally, the MAP3K Tpl2 activates ERK in response to proinflammatory stimuli (5).

The activation of ERK by proinflammatory stimuli may play important roles in innate immune responses and autoimmunity. In addition to its well-characterized role in mitogenic signaling, ERK is activated in various cell types by proinflammatory cytokines such as TNF and IL-1 (6, 7). ERK can also be activated by IgG-containing immune complexes through FcγRII/RIII in neutrophils, monocytes, and macrophages (8, 9). Inhibitor studies suggest that ERK activation may play a role in FcγR-stimulated neutrophil chemotaxis and cytokine production by monocytes. It is not known, however, whether ERK activation is important for inflammatory processes in vivo (10, 11).

Rheumatoid arthritis is one such process for which the mechanism of joint destruction is incompletely characterized. Several mouse models exist to study the pathogenesis of arthritis. The passive transfer model of autoimmune arthritis uses serum from the K/BxN TCR transgenic mouse, which contains Abs against ubiquitously expressed glucose 6-phosphate isomerase, to induce transient arthritis in normal recipient mice (12, 13). This mechanism of disease induction requires only components of the innate immune system (14–23), including macrophages, neutrophils, and the proinflammatory cytokines TNF and IL-1 (18, 24–26).

Recently, it has become clear that scaffold proteins play important roles in the activation of MAPK. Although the exact function of MAPK scaffolds is not clear, they are believed to regulate the local concentration of components of the MAPK pathway as well as direct MAPK subcellular localization (27). The best-studied ERK MAPK scaffold is kinase suppressor of Ras (KSR)3, which was originally cloned in C. elegans and Drosophila as a positive regulator of the Ras/MAPK signaling pathway. By binding to Raf, Mek, and Erk, KSR facilitates their activation in response to growth factor stimulation and AgR cross-linking (28–30). A second isoform, KSR2, was recently cloned and is implicated in the activation of MEK and ERK by the MAP3Ks Tpl2 and MEKK3 (31, 32).

Although KSR1 is broadly expressed in tissues such as brain, testes, thymus, and spleen, the expression of KSR1 in cells of the innate immune system is unknown. Similarly, little is known about the role of KSR1 in Erk activation by proinflammatory stimuli. In this study, we tested whether KSR1 was involved in ERK activation by proinflammatory and stress stimuli. To determine whether KSR1 plays a role in a physiological inflammatory process in vivo, we compared wild-type mice and KSR1-deficient mice in susceptibility to a mouse model of arthritis.

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3 Abbreviation used in this paper: KSR, kinase suppressor of Ras; MEF, mouse embryonic fibroblast.
Materials and Methods

Generation of mouse embryonic fibroblasts (MEF)

MEFs were derived from day 13.5 embryos. The head and internal organs were removed, and embryos were minced in trypsin-EDTA. Trypsin digests were continued at 37°C until tissue was disaggregated. Trypsin was inactivated with DMEM containing 10% FCS and cells were centrifuged at 150 × g for 8 min. Pellets were resuspended in DMEM containing 10% FCS, 2 mM L-glutamine, 0.1 mM nonessential amino acids, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were plated on 150-mm tissue culture dishes and grown until confluent at 37°C in 10% CO₂. For immunoblotting, MEFs were plated at 5E5 cells per well in 6-well plates. After 24 h, MEFs were stimulated with 10 ng/ml TNF or 10 ng/ml IL-1, which are both gifts from Dr. R. Schreiber (Washington University School of Medicine, St. Louis, MO) in the same medium used for culturing, then were harvested for analysis.

Generation of bone marrow-derived macrophages and neutrophils

To generate macrophages, bone marrow was flushed from femurs of wild-type and KSR1-deficient mice with RPMI 1640 containing 10% FBS, using a 5-ml syringe and a 25-gauge needle. Cells were plated on 150-mm bacteriological petri dishes at a concentration of 10⁶ cells per plate. Cultures were grown for 6 days in DMEM supplemented with 20% FBS and 20% L929 cell-conditioned medium. Confluent cultures were trypsinized and resuspended in 6-well plates for Western blotting. Before stimulation with 10 ng/ml LPS, macrophages were cultured overnight in DMEM supplemented only with 0.5% FBS.

To generate neutrophils, bone marrow was flushed from femurs of wild-type and KSR1-deficient mice with HBSS containing 1% FBS. Neutrophils were enriched by centrifugation over a Percoll gradient, then were stained with Abs to Gr-1 and CD11b (BD Biosciences), and cells positive for both markers were sorted using a FACSVantage SE (BD Biosciences). Resulting cells were ~93% neutrophils.

Transfection and immunoprecipitation

HEK 293 cells were grown on 6-well plates in DMEM supplemented as described. Cells were transfected with Superfect (Qiagen) using 2 μg of plasmid DNA and 8 μl of Superfect per well. Full-length KSR1, the N terminus of KSR (residues 1–539), the C terminus of KSR (residues 540–873), or the CA4 domain of KSR1 (residues 378–539), with a C-terminal Flag tag, were expressed from pCMV5. MEKK1 was expressed in pCMV5, which is a gift from Dr. K. Blumer (Washington University School of Medicine, St. Louis, MO), after adding an N-terminal Myc tag. Cells were harvested 36 h after transfection and were lysed in buffer containing 50 mM Tris-Cl (pH 8.0), 150 mM NaCl, 0.5% Nonidet P-40, 2.5 mM sodium pyrophosphate, 5 mM EDTA, 5 mM EGTA, 2 mM sodium orthovanadate, 5 mM sodium fluoride, 2 mM PMSE, 20 μM leupeptin, and 20 μM pepstatin. Lysates were clarified by centrifugation at 10,000 × g for 10 min at 4°C. Supernatants were precleared by incubation with preimmune serum and protein A-Sepharose (Sigma-Aldrich) for 30 min. Immunoprecipitation was performed using polyclonal anti-Myc or anti-MEKK1 (C-22; Santa Cruz Biotechnology) overnight. Protein A-Sepharose was then added for 1 h. Complexes were washed in lysis buffer, then resolved by SDS-PAGE on 10% polyacrylamide. Proteins were transferred to nitrocellulose, and immunoblots were performed with anti-MEKK1 (1/2,500) or monoclonal M2 anti-FLAG (1/10,000) (Sigma-Aldrich). Secondary donkey anti-rabbit or goat anti-mouse HRP-conjugated secondary Abs (Jackson ImmunoResearch Laboratories) were used at 1/10,000 (see Fig. 2A). HRP was detected using an ECL kit (Pierce). Goat anti-rabbit or anti-mouse infrared dye-conjugated Abs (LI-COR) were used at 1/10,000 (see Fig. 2B), followed by imaging of the blot on a LI-COR Odyssey.

Phospho-ERK immunoblotting

MEFs were lysed as described. Following SDS-PAGE on 10% polyacrylamide, proteins were transferred to nitrocellulose. Immunoblotting was performed with polyclonal anti-phospho-Erk1/2 (Cell Signaling Technology) used at a 1/2,000 dilution, or polyclonal anti-Erk2 (Santa Cruz Biotechnology) used at a 1/2,500 dilution. Secondary HRP-conjugated donkey anti-rabbit Ab (Jackson ImmunoResearch Laboratories) was used at a 1/10,000 dilution. HRP was detected using an ECL kit (Pierce).

RT-PCR

RNA was isolated from brain, thymus, spleen, bone marrow-derived macrophages, and neutrophils using TRIzol (Invitrogen Life Technologies) according the manufacturer’s instructions. Two micrograms of total RNA was used to synthesize cDNA using random hexamer primers (Invitrogen Life Technologies). cDNA was screened for KSR1 expression using primers and probes generated by Assays-by-Design (Applied Biosystems). 18 S RNA was quantitated using predeveloped TaqMan assay reagents (Applied Biosystems). PCR was performed on an ABI PRISM 7000 (Applied Biosystems). KSR1 expression levels in various tissues were compared with brain, the expression level of which was set to 1.

Induction of arthritis

The 8- to 10-wk-old KSR1-deficient and wild-type DBA/1Lacj littermate mice were injected i.p. with 50 μl of serum from arthritogenic KRN transgenic mice. Ten mice of each genotype were injected. Every 24 h, mice were scored visually for signs of inflammation, and hind ankles and fore paws were measured with calipers to quantitate swelling. Each limb was assigned a clinical index on a five-point scale, as follows: 0) no visible inflammation; 1) doubt; 2) mild swelling or redness; 3) severe swelling of ankle; and 4) severe swelling of ankle and involvement of other joints. Swelling of limbs was quantitated by subtracting the day-0 measurement taken at the time of injection from each subsequent measurement. Statistically significant was determined using the two-tailed Student’s t test. Animal protocols were reviewed and approved by the Washington University Animal Studies committee.

Results

KSR1-deficient fibroblasts exhibit a defect in ERK activation by osmotic shock

To determine whether KSR regulates ERK activation by stress stimuli, we derived MEF from heterozygous and KSR knockout animals. We first tested cellular responses to osmotic shock by treating cells with sorbitol for various time periods. ERK activation was assessed by immunoblotting cell lysates with a phospho-specific Ab to activated ERK. Although untreated cells demonstrated robust ERK activation after both sorbitol and 2C11 treatment (Fig. 1A, left), ERK activation in KSR-deficient cells was decreased in both intensity and duration (Fig. 1A, right). Stripping and reprobing the same blots with an Ab recognizing ERK2 demonstrated that similar amounts of protein were present in all lanes. Thus, the activation of ERK by osmotic shock is facilitated by KSR1.

KSR is known to be required for efficient ERK activation via the MAP3K Raf (28, 29, 33). However, sorbitol activation of ERK is thought to use MEK1 and not Raf-1 (4). To confirm that sorbitol-mediated ERK activation was, in fact, Raf-independent, cells were treated with a Raf kinase inhibitor (GW5074) before treatment with sorbitol or with 2C11, a CD3 cross-linking Ab known to activate Erk through Raf-1. Although untreated cells demonstrated robust ERK activation after both sorbitol and 2C11 treatment (Fig. 1B, lanes 2, 5, 6, and 7), the Raf kinase inhibitor reduced ERK activation only in response to 2C11 (Fig. 1B, lane 3), and had no effect on sorbitol activation of ERK (Fig. 1B, lanes 8, 9, and 10). This finding confirmed that the sorbitol signaling pathway activates ERK independently of Raf-1.

KSR1 and MEK1 exist in a complex when overexpressed

The inability of a Raf kinase inhibitor to block Erk activation by sorbitol suggested that KSR may interact with another MAP3K, in addition to Raf. Because sorbitol stimulation involves the MAP3K MEKK1, we tested whether MEK1K interacts with KSR. HEK 293 cells were transfected with expression plasmids for an epitope-tagged KSR1 alone or together with MEK1K. MEK1K immunoprecipitates were analyzed for the presence of KSR by immunoblotting for the FLAG epitope. KSR was easily detectable in the MEK1K immunoprecipitate (Fig. 2A, lane 3) when both were expressed together, whereas only a small amount of KSR1 was co-precipitated when MEK1K was not expressed (Fig. 2A, lane 2). This result probably represents a small amount of nonspecific binding of the MEK1K Ab to KSR1. Nevertheless, these results support the idea that MEK1K binds to KSR1.
To verify the specificity of binding, we tested fragments of KSR1 for their ability to bind MEKK1. Constructs containing the N terminus of KSR1 or the C terminus of KSR1 were tested for binding to MEKK1. Both the N-terminal and C-terminal fragments of KSR1 coprecipitated with MEKK1, suggesting that, similar to KSR1 binding to Raf-1, multiple binding sites for MEKK1 exist (Fig. 2B, lanes 6 and 7). We next generated a construct deleting sequences encoding residues 1–378 of the N-terminal fragment of KSR1. This truncated construct, KSR-C4A (378–539), was not detectable in the MEKK1 immunoprecipitate (Fig. 2B, lane 8), suggesting that the N-terminal binding site is contained within residues 1–378 of KSR1.

KSR1-deficient cells exhibit a defect in ERK activation following stimulation with proinflammatory cytokines or LPS

To test whether KSR regulates ERK activation by other stress stimuli, such as proinflammatory cytokines, MEF derived from heterozygous and knockout mice was stimulated with TNF or IL-1 for various times. Lysates were analyzed for ERK activation by immunoblotting. Similar to results obtained with TNF and IL-1, both the intensity and duration of ERK activation by LPS were reduced in the absence of KSR1 (Fig. 3C). Reprobing the blots with an Ab recognizing ERK2 (Fig. 3, A and B, bottom panels) or p38 (Fig. 3C, lower panel) demonstrated that similar levels of protein were loaded in all lanes.

KSR1-deficient mice are resistant to Ab-induced arthritis

Because ERK activation by proinflammatory cytokines was attenuated in KSR1-deficient cells, we were interested in determining
**Figure 3.** ERK activation by TNF, IL-1, and LPS is impaired in the absence of KSR1. KSR1 heterozygous (Het) or knockout (KO) fibroblasts were treated with 20 ng/ml TNF (A) or 20 ng/ml IL-1β (B). KSR1 wild-type or knockout macrophages were treated with 10 ng/ml LPS (C). Cells were lysed at time points between 0 and 150 min. Lysates were separated by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with a phospho-specific ERK1/2 Ab (top panels). Immunoblotting with an ERK2 Ab (bottom panels) demonstrated similar levels of loading in all lanes. Results are representative of three experiments.

**Discussion**

KSR was originally identified as a positive effector in the Ras signaling pathway (30, 35, 36). Mutated alleles of KSR suppress the phenotypes caused by activated Ras, restoring the *Drosophila* roughened eye and *C. elegans* multivulva to normal (30, 35, 36). These mutations in KSR did not suppress a similar roughened eye phenotype resulting from active Raf mutations (30). As a result, KSR was thought to act between Ras and Raf to facilitate MAPK activation (30). Deletion of KSR in mice was later shown to impair MEK activation in response to mitogenic stimuli, further supporting its role as a facilitator for Ras/Raf signaling (29). The observations that KSR1 associates with ERK, MEK, and Raf led to the proposal that KSR is a scaffold for the Ras/Raf pathway (28, 29, 37–39).

In this study we have shown that KSR1 facilitates ERK activation by stress and proinflammatory stimuli. Fibroblasts exposed to osmotic stress activated ERK in a KSR1-dependent manner. Similarly, in both fibroblasts and macrophages treated with inflammatory stimuli such as TNF, IL-1, and LPS, ERK activation was dependent on KSR1 for maximal intensity and duration.

Recently a second isoform of KSR was cloned. The C-terminal halves of KSR1 and KSR2 exhibit ~80% homology, suggesting that they will have similar functions in vivo. Indeed, preliminary evidence suggests that KSR2 is also capable of binding to Raf, MEK, and ERK (32). It will be important to determine the expression pattern of KSR2 and determine whether its functions are distinct from, or overlap with, those of KSR1. Current evidence suggests that KSR2 may be involved in ERK activation by the MAP3Ks Tpl2 and MEKK3 (31, 32).

Our observation that KSR1 associates with MEKK1 indicates that rather than being restricted to Raf, KSR may bind other MAP3Ks and facilitate ERK activation by a wide variety of stimuli. Because the ability of Raf to efficiently activate MEK and ERK appears to depend on its interaction with KSR1 (28), it is not surprising that MEKK1 appears to demonstrate a similar requirement. We were, unfortunately, unable to observe an association between endogenous KSR1 and endogenous MEKK1. This may be due to the low expression levels of KSR1 in most tissues. Alternatively, it may be caused by a low level of constitutive association between KSR1 and MAP3K. Although KSR1 associates constitutively with MEK, its association with Raf is dependent on external stimuli (33, 37, 39, 40). Finally, because KSR1 is also a scaffold for Raf, it is possible that different pools of KSR1 exist within a cell, with only a fraction of KSR1 available for binding to a particular MAP3K even under conditions that facilitate association. Interestingly, MEKK1 appears to associate independently with the N terminus and C terminus of KSR equally. This observation is perhaps not surprising as there are multiple independent binding sites for the MAP3K Raf-1 on KSR1 (33). In the N-terminal domain binding appeared to be mediated by the first 378 aa because a construct lacking these residues (KSR1-CA4) did not bind.

It is not known whether KSR1 scaffolds ERK to all MAP3Ks capable of ERK activation, or whether KSR1 only cooperates with a subset of MAP3K. We are currently testing the interaction between KSR1 and other MAP3Ks, and investigating the correlation between KSR1 binding and the ability of MAP3K to activate ERK. The requirement for KSR1 may depend on the stimulus, the subcellular localization of the MAP3K, or it may be a general requirement for all ERK activation. Because the activation of ERK by LPS, TNF, and IL-1 is thought to be mediated by another MAP3K, Tpl2, it will be important to determine whether KSR1 interacts with Tpl2. A recent report shows that Tpl2 interacts with KSR2 (32). Binding is mediated by the C terminus of KSR2, which is highly homologous to the C terminus of KSR1. Given the phenotype of the Tpl2 knockout mouse (5) and our observed impairment of ERK activation by LPS and TNF in KSR1-deficient cells, we anticipate that KSR1 will also bind Tpl2. It is interesting to speculate that KSR1 and KSR2 may differ in their ability to bind...
MAP3K. The inflammatory phenotype of KSR1-deficient cells is less drastic than that of Tpl2 knockout cells, so perhaps KSR2 compensates for the loss of KSR1 in macrophages. Alternatively, KSR1 and KSR2 may have different binding specificities and mediate different aspects of ERK signaling.

KSR1 expression in tissues of the innate immune system suggests that it may play a role in processes of innate effector functions such as inflammation and autoimmune reactions. Rheumatoid arthritis is a chronic and debilitating disease affecting millions of individuals worldwide (reviewed in Ref. 41). Several small-animal models have been developed to study the progression of disease. The most common rodent model of rheumatoid arthritis is collagen-induced arthritis, in which mice or rats are immunized with type II collagen to produce an autoimmune response (reviewed in Ref. 42). Susceptibility to collagen-induced arthritis depends on the normal function of T and B cells, which initiate the immune response (14, 15). Previous work from our laboratory demonstrated that KSR1-deficient T cells were functionally impaired (29). To examine the role of KSR1 in diseases mediated by the innate immune system it was necessary to use a model that bypassed the adaptive immune response.

In the passive transfer model of rheumatoid arthritis, serum from K/BxN TCR transgenic mice is transferred to normal recipients (12). Abs against glucose 6-phosphate isomerase form immune
complexes, which are deposited on cartilage surfaces, and activate monocytes, macrophages, and neutrophils through the Fc receptors FcγRI and FcγRIII (16, 43–45). Several lines of evidence suggest that ERK activation may be involved in the pathogenesis of arthritis at this point. First, the activated cells of the innate immune system produce proinflammatory cytokines, which can activate ERK (8, 9). Second, inhibitor studies have suggested that ERK activation is important in FcγR-stimulated neutrophil chemotaxis and cytokine production by monocytes (10, 11). However, to date no compelling evidence has been shown in vivo for ERK involvement in the progression of autoimmune diseases such as rheumatoid arthritis. In this study we demonstrated that KSR1-mediated ERK activation is required for the progression of joint damage in passively transferred arthritis.

It will be important to elucidate the mechanism by which ERK mediates the development of arthritis in mice receiving arthritogenic serum. The observation that ERK activation by proinflammatory stimuli is absent in the absence of KSR1 in vitro, and that the severity of arthritis is ameliorated in vivo suggests that KSR1-mediated ERK activation is an important effector mechanism in the pathogenesis of arthritis. KSR1 is expressed in several cell types known to be relevant to the development of arthritis. It will be important to define the role of ERK in the effector functions of these cells. The high level of expression of KSR1 in neutrophils is especially noteworthy. Because TNF and IL-1 can stimulate the activation of ERK in neutrophils (26), neutrophils lacking KSR1 may be less responsive to proinflammatory cytokines, thereby creating resistance to arthritis.

In conclusion, we have established that KSR1 can serve as a scaffold for MAP3Ks other than Raf, facilitating ERK activation by stress and proinflammatory stimuli. In the absence of KSR1, susceptibility to autoimmune arthritis is reduced. It will be important to determine the extent of involvement of KSR in ERK activation, and to explore the effects that deletion of KSR will have on susceptibility to disease.

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Disclosures
The authors have no financial conflict of interest.

References

FIGURE 5. KSR1 is expressed in macrophages. Expression of KSR1 was quantified by real-time RT-PCR using the TaqMan fluorogenic probe system, and normalized to 18 S mRNA. Data are expressed relative to the average of two independent experiments is shown, with error bars representing one SD.